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# STUDIES ON PENETRATION OF DYES WITH GLASS ELECTRODE

## IV. PENETRATION OF BRILLIANT CRESYL BLUE INTO *NITELLA FLEXILIS*

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(Accepted for publication, June 19, 1930)

### I

#### INTRODUCTION

Although basic dyes play a special rôle in biology, because many of them are readily taken up by living cells, the form in which they penetrate has never been satisfactorily determined.

A basic dye is usually obtained in the form of the salt some of which may be transformed in aqueous solution to free base (the amount depending on the "apparent" dissociation constant of the dye and the pH value), and in the range of pH values generally employed with living cells (approximately between pH 5 and 9.5) both forms of dye must be considered. In this range methylene blue, for example, exists only as a salt. It is very strongly basic<sup>1</sup> so that even at pH 9.5 no free base is formed. Lauth's violet, on the other hand, is less basic, with a basic dissociation constant<sup>1</sup> of  $1.9 \times 10^{-3}$ .

Since the nature of the transformation of the dye salt into the free base is still in dispute<sup>2</sup> the structure of the salt and free base of the dye will not be discussed.

Furthermore, the dissociation constant of cresyl blue has not been determined by such methods as the oxidation-reduction potential or the glass electrode, so that the basicity of the dye is not accurately known.

It is, however, possible to determine approximately the relative ratio of the dye in form of free base/salt by shaking the chloroform with the dye solution and de-

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<sup>1</sup> Clark, W. M., Cohen, B. C., and Gibbs, H. D., *Hygienic Laboratory Bulletin*, 1928, No. 151, 202-206.

<sup>2</sup> Henrich, F., *Theories of organic chemistry*, translated by John Johnson and Dorothy Hahn, London, 1922.

termining colorimetrically the "apparent" partition coefficient  $k_{ap} = \frac{C_1}{C_2}$ , where  $C_1$  is the concentration of free base of the dye in chloroform and  $C_2$  the concentration of the total dye (free base in equilibrium with the salt) in the aqueous solution. Such a determination was previously made<sup>3</sup> and it was found that the values of the  $k_{ap}$  increased with rise in the pH values as if the basic dissociation constant of the dye were about  $10^{-5.6}$ . But owing to the extreme solubility of the free base in chloroform and to the progressive alteration in the color of the dye in the aqueous solution from about pH 10 upward, it was not possible to determine the value of the true partition coefficient  $k_{tr} = \frac{C_3}{C_4}$  where  $C_3$  represents the concentration of free base of the dye in chloroform and  $C_4$  the same in the aqueous solution. The pH value at which the dye existed almost entirely in form of free base was assumed and the value of  $k_{tr}$  was accordingly calculated. The dissociation constant was then calculated on basis of this assumption so that it may prove to be different when the determination is made with a more reliable method. But the significance of this observation does not lie in the accurate determination of the dissociation constant but in the proof that the relative increase in the values of  $k_{ap}$ , showing roughly the relative ratio of free base/salt, more or less corresponds to the relative increase in the rate of penetration of the dye into *Nitella* as the pH value of the aqueous dye solution is raised.

It has been suggested that basic dyes penetrate living cells in the form of free base<sup>4</sup> and not in the form of salt,<sup>5</sup> but this, despite many attempts, has never been really proven. The rate of penetration of a basic dye has been found to increase with a rise in the pH value of the external dye solution. This observation might be interpreted as showing penetration in the form of free base since the concentration of this form of dye increases as the external pH value rises. But it might be held to indicate that the dye penetrates as positive ions since with a rise in the external pH value an increase of the negative charges in the cell surface might occur which might result in greater penetration of the positive dye ions.

<sup>3</sup> Irwin, M., *Proc. Soc. Exp. Biol. and Med.*, 1925-26, 23, 251; *J. Gen. Physiol.*, 1925-26, 9, 561.

<sup>4</sup> The following authors assume that basic dyes penetrate as free base. Overton, E., *Jahrb. wissenschaft. Bot.*, 1900, 43, 669. Harvey, E. N., *J. Exp. Zool.*, 1911, 10, 507. Robertson, T. B., *J. Biol. Chem.*, 1908, 4, 1. McCutcheon, M., and Lucké, B., *J. Gen. Physiol.*, 1923-24, 6, 501. Irwin, M., *J. Gen. Physiol.*, 1925-26, 9, 561.

<sup>5</sup> The following authors account for the penetration of basic dyes through combining of dye cations with protoplasmic constituents. Bethe, A., *Biochem. Z.*, 1922, 137, 18. Rohde, K., *Arch. ges. Physiol.*, 1920, 182, 114. Pohle, E., *Deutsch. med. Wochn.*, 1921, 47, 1464. Collander, R., *Jahrb. wissenschaft. Bot.*, 1921, 60, 354.

If the dye penetrates as free base and combines<sup>6</sup> with the sap to form salt which cannot readily escape from the vacuole, an increase in the pH value of the sap might be expected to decrease the rate of penetration. When the pH value of the sap of *Nitella* was increased by entrance of ammonia, the rate of penetration of cresyl blue was decreased<sup>7</sup> from which it was concluded<sup>7</sup> that the dye penetrated as free base. If such a result were indicative of the penetration of the dye as free base we should expect that in decreasing<sup>8</sup> the pH value of the sap an increase in the rate of penetration of cresyl blue would take place. But on lowering the pH value of the sap, through the entrance of acetic acid, a decrease<sup>8</sup> in the rate of penetration was observed, instead of an increase. This decrease may be due to an inhibitory effect of the acid on the protoplasm irrespective of the changes in the sap since various<sup>9</sup> other substances produce an inhibiting effect without penetrating the vacuole.

It is therefore desirable to find a satisfactory way of distinguishing between the penetration of the free base and that of the dye salt. This can be done by observing the effect of the dye on the pH value of the sap. The hydrogen electrode cannot be utilized, owing to the poisoning of the electrode by the dyes and to their oxidation-reduction potential, and the glass electrode is therefore employed. Since this requires the use of about 0.4 cc. of sap it is necessary to choose large cells which yield sap in sufficient amount for analysis. This can easily be collected from the vacuoles of cells of *Nitella*. Its behavior toward dyes is roughly comparable to that of such cells as *Paramecium*, *Spirogyra*, *Elodea*, and *Valonia* so that by studying the mechanism of dye penetration with *Nitella* we may gain some insight into the behavior of other cells as well.

In the present paper therefore determinations of the pH values of the sap of *Nitella* by the glass electrode<sup>10, 11</sup> are used as a means of studying the form of dye penetrating the vacuoles of living cells.

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<sup>6</sup> E. N. Harvey was the first to suggest that the free base was converted to the salt in the sap. See reference under footnote 4.

<sup>7</sup> McCutcheon, M., and Lucké, B., *J. Gen. Physiol.*, 1923-24, 6, 501.

<sup>8</sup> Irwin, M., *Proc. Soc. Exp. Biol. and Med.*, 1926-27, 24, 54; *J. Gen. Physiol.*, 1926-27, 10, 271; 1927-28, 11, 111.

<sup>9</sup> See footnote 8, and also Irwin, M., *J. Gen. Physiol.*, 1926-27, 10, 425.

<sup>10</sup> Through the kindness of Dr. D. A. MacInnes and of Dr. M. Dole a glass electrode apparatus was devised with which it was possible to measure a very small amount of liquid. Through their cooperation it was possible to determine whether this method was applicable to the studies of the penetration of cresyl blue into *Nitella* cells, as mentioned in their article (MacInnes, D. A., and Dole, M., *J. Gen. Physiol.*, 1928-29, 12, 805).

<sup>11</sup> Applicability of the glass electrode methods to the measurement of the sap, mentioned under footnote 9, was described separately by Irwin, M., *Proc. Soc. Exp. Biol. and Med.*, 1929-30, 27, 132. Preliminary report of the present paper was published, *Proc. Soc. Exp. Biol. and Med.*, 1929-30, 27, 991.

## II

*Methods*

*Solutions Employed.*—All buffer solutions were made up according to Clark's<sup>12</sup> standards and diluted ten times. The pH value of these solutions was checked by means of the glass electrode. The dyes were in all cases dissolved in these dilute solutions.

*Condition of the Dye Used.*—The cresyl blue was purchased as a salt. The brilliant cresyl blue (called cresyl blue in the text) was manufactured by Grüber previous to 1914, and appears to contain (in the range of pH values between pH 5.5 and pH 9.2) chiefly cresyl blue in form of salt or free base. It also contains another dye, *X*, but in the solutions employed the amount is so small in comparison to the cresyl blue (free base or salt) that *X* may be neglected. The difference in behavior between the cresyl blue and *X* is well marked so that it can be readily distinguished. Cresyl blue is absorbed as free base by the chloroform and appears orange. This orange dye can be entirely removed from the chloroform on shaking the chloroform with the artificial sap of *Nitella* (0.1 N KCl at pH 5.5) in which it appears blue. On shaking the cresyl blue solution with chloroform, the dye is absorbed much more from pH 9.2 than from pH 5.5, showing that the free base of cresyl blue is much more soluble in chloroform than the dye salt, and that the ratio of free base/salt of cresyl blue increases with the rise in the pH value. The dye *X*, on the other hand, appears pinkish red in chloroform. The amount of *X* absorbed by the chloroform is so much less than that of the free base of cresyl blue that it becomes visible only when the experimental condition is favorable (*i.e.*, at a high concentration and low pH value). For example, on shaking 0.07 per cent cresyl blue solution at pH 5.5 with the chloroform, the dye appears red in chloroform. On shaking with artificial sap, the red dye is not removed from the chloroform; in fact only a trace of the blue dye appears in the sap, which may very well be a trace of the cresyl blue extracted from the chloroform. On shaking 0.005 per cent cresyl blue solution at pH 9.2 or at pH 5.5, or 0.07 per cent cresyl blue solution at pH 9.2, or 0.025 per cent cresyl blue solution at pH 6.8 with chloroform, the dye absorbed appears orange in chloroform. But when this chloroform is shaken with the sap, until the orange dye is completely removed from the chloroform, the red dye becomes visible in the chloroform, and is not extractable by the sap from the chloroform. This red dye is absorbed by the chloroform to about the same extent from pH 9.2 or from pH 5.5 cresyl blue solution, but the amount absorbed increases with the rise in the concentration of aqueous cresyl blue solution. The amount of *X* absorbed is so small, however, that the absorption causes only a very slight decrease in the concentration of the total dye solution

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<sup>12</sup> Clark, W. M., The determination of hydrogen ions, Baltimore, 3rd edition, 1928.

even when the chloroform is shaken with 0.07 per cent cresyl blue solution at pH 5.5. These experiments are being continued and their results will be reported in later publications.

Spectrophotometric measurements of these solutions of cresyl blue show that they give absorption curves characteristic of cresyl blue.

The free base of cresyl blue was obtained as follows: 0.005 per cent cresyl blue solution at pH 9.2 was shaken with chloroform. The chloroform was then carefully freed from the aqueous solution and immediately placed in a pyrex glass vial and caused to evaporate rapidly by bubbling air through the solution. The freshly collected sap was then placed in this glass vial, and the concentration of the dye was determined colorimetrically by comparison with the standard dye solutions of known concentrations. There was a small amount of the dye residue which was not readily soluble in the sap, and which was therefore discarded. The pH value of the sap containing the dissolved dye was at once determined by the glass electrode.

To ascertain whether this dye was contaminated by other alkaline substances absorbed by the chloroform from the buffer solution which might alter the pH value of the sap the following experiments were made. The chloroform was shaken with the buffer solution at pH 9.2 containing no dye and was evaporated in the vial as before. The freshly extracted sap was placed in the vial and the pH value was immediately determined by the glass electrode. Since the pH value of the sap collected in this vial was the same as that of the sap collected in a control vial (which had not come in contact with the chloroform) it is apparent that the chloroform does not absorb any alkaline substance from the buffer solution in the absence of dye which would alter the pH value of the sap. Whatever alteration occurs in the pH value of the sap in presence of the dye must therefore be due to the dye alone.

It may be asked whether this dye is actually free base of cresyl blue. It cannot be a pseudo base, since it is very soluble in water and is readily reversible, forming a salt at lower pH values (appearing blue), while the pseudo base is not readily soluble in water, in which it appears colorless, and is irreversible. On shaking this dye solution (made up in buffer mixtures) with chloroform, the behavior is found to be very much like that of the original cresyl blue solution. The spectrophotometric determination shows that the absorption curve of the dye taken up by chloroform is identical with that of brilliant cresyl blue. It would therefore appear that the dye represents primarily the free base of brilliant cresyl blue. A detailed account of these results will be subsequently published.

*Condition of Cells before Experiments.*—As previously described,<sup>13</sup> single cells were cut from the central portion of the plant and kept about 20 hours in pans of

<sup>13</sup> Regarding the care of cells and detection of injury see Irwin, M., *J. Gen. Physiol.*, 1925-28, 8, 147; 1926-27, 10, 271, 927; 1927-28, 11, 123; 1928-29, 12, 147.



tap water at about 20°C. in a well ventilated room in diffused light. The cells which have suffered severe injury at the time of cutting die over night and those which may be slightly injured may recover so as to be suitable for use. The effect of cutting and of the subsequent treatment of the cells as described in connection with electrical measurements<sup>14</sup> is not manifested in the same way in the determination of penetration of dyes into the vacuole, so that it is not necessary, for example, to keep each cell in a separate test-tube for a day or more before an experiment. The precaution, however, was always taken of placing only a few cells in a large pan of water so as to minimize any possible effect of the sap diffusing from the dead cells to the living cells.

*Experiments on Penetration.*—Cells were placed in solutions with one end (only a very small portion, just enough to avoid contamination) exposed to the air in a moist chamber, at the temperature at which the glass electrode measurement was subsequently made. The cells were kept in the dye solution about 15 minutes. Leaving one end of the cell out of the solution for this period in a moist chamber was not injurious since cells kept in this way in tap water containing no dye even after 8 hours showed no signs of injury.

*Extraction of the Sap.*—The cells were removed from the solution, rinsed rapidly in distilled water and wiped gently first with a soft dampened cheese cloth and then with the dry cloth. Then each cell was cut open by a pair of scissors (the cut being made at the end which projected out of the solution) and the sap was allowed to flow out into a pyrex glass vial (small enough to prevent evaporation). By thus extracting the sap from the unstained portion of the cell, it was possible to avoid contamination of the sap by dye diffusing out of the cell wall at the time of extraction. Two successive extractions of sap were made from the vacuole of each living cell. The first extraction, called for convenience "sap," was made without pressing on the cell wall, while the second extraction called "sap mixture" was made by squeezing the cell wall vigorously so as to press the protoplasm into the vacuolar sap. The pH value of the sap was found to be about 0.6 pH lower than that of the sap mixture. It took about 15 minutes for one person to collect the sap and the corresponding sap mixture sufficient for analysis. For each reading by the glass electrode method it required about 0.4 cc. of sap collected from the vacuoles of 40 or more cells. The extraction was made at the same temperature as the temperature at which the electrode measurement was made.

*Standardization of the Glass Electrode and Method of Measurement.*—The apparatus used is identical with the one described by MacInnes and Dole<sup>15</sup> but the method of standardization of the glass electrode and the calculation of the pH value of the unknown solution used was different. The standard solution of the M/20 potassium hydrogen phthalate was made up to pH 3.97 and its pH value was determined by means of the hydrogen electrode. This solution was made up every

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<sup>14</sup> Osterhout, W. J. V., and Hill, S. E., *J. Gen. Physiol.*, 1929-30, 13, 467.

<sup>15</sup> For reference see footnote 10.

2 weeks to keep the pH value constant. Due to the very low temperature coefficient of this solution the alteration in the pH value with varying temperature is negligible in the range in which the experiments were carried out (between 20° and 23°C.).

Before each series of experiments the temperature was noted, and the glass electrode was standardized by determining the potential of the standard phthalate solution<sup>16</sup> at pH 3.97. Then the pH value of the standard M/15 phosphate buffer solution at pH 6.647 (the pH value of which was checked by the hydrogen electrode) was determined by means of the glass electrode to ascertain the behavior of the electrode; in no case did the reading vary more than  $\pm 0.03$  pH. The potential of the unknown solution was then determined, and the pH value of the unknown solution was calculated for the given temperature according to the usual formula<sup>17</sup>

$$\text{pH}_x = \text{pH}_s + \frac{E_x - E_s}{\frac{RT}{F}}$$

where  $\text{pH}_x$  denotes the pH value of the unknown solution and  $\text{pH}_s$  that of the standard, while  $E_x$  represents the potential of the unknown solution and  $E_s$  that of the standard (the value of  $\frac{RT}{F}$  at different temperatures is given by Clark<sup>11</sup>).

The temperature during the day did not usually vary more than  $\pm 0.5^\circ\text{C}$ . but in case a variation greater than  $\pm 0.5^\circ\text{C}$ . occurred, the electrode was re-standardized. From day to day the temperature varied between 20° and 23°C., but this variation did not bring about errors, since the glass electrode was always standardized and a temperature correction was made accordingly. After each series of experiments the electrode was again standardized with the phthalate and the phosphate buffer to see whether the electrode remained unaltered during the experiments. In many cases where the test solution is much less buffered than the standard phosphate solution, it may be desirable to check the glass electrode before and after a series of experiments with a standard solution which is buffered to about the same extent as the test solution. This was done in the case of the *Nitella* sap, by using M/150 phosphate buffer solution at pH 5.5, or the control sap, whenever there was a possibility of the effect of the sap in presence of the dye on the electrode in a series of experiments. In the case of all the experiments presented in the text the electrode remained unchanged through each series of experiments, in that there was no variation of more than  $\pm 0.05$  pH value with these standard solutions.

<sup>16</sup> For review and bibliography on the glass electrode methods see Clark, W. M., The determination of hydrogen ions, Baltimore, 3rd edition, 1928.

<sup>17</sup> The equation is taken from the one described by Kerridge, P. T., *Biochem. J.*, 1925, 19, 611.

Three electrodes were employed for the experiments described in the text. In no case did the pH value of the standard phosphate buffer solutions determined by these electrodes vary more than  $\pm 0.03$  pH.

*Method of Washing the Glass Electrode when Successive Determinations Are to be Made.*—When the glass electrode is not in use, it is kept in 0.1 N HCl. When two measurements with different samples are to be made in rapid succession washing with distilled water (without washing with the acid) is adequate if the test solution is such that it does not measurably affect the electrode or the reading, or if the solution is sufficiently buffered, but if the test solution does affect the electrode and if it is not sufficiently buffered, the alteration will result in a wrong pH value. An example of such cases may be cited as follows. The pH value of a strongly buffered solution, such as standard phosphate buffer at pH 6.647, in presence or absence of basic dye, or that of the distilled water containing no dye, may be measured repeatedly without showing any effect of the test solution on the electrode whether the electrode is washed after each measurement with 0.1 N HCl and then with distilled water or only with distilled water. But if the distilled water contains, for example, 0.07 per cent cresyl blue in form of salt or free base, an effect on the electrode is manifested by an increase in the pH value after each successive measurement if the electrode is washed only with distilled water each time. But such an effect is not manifested if the electrode is first washed with acid each time before applying distilled water.

The freshly extracted sap is more buffered than the distilled water but not sufficiently so to enable us to omit the washing with acid each time, even though the electrode is always washed with the test sap before each measurement. If the electrode is not washed with the acid there is a small but progressive increase in the observed pH value of sap containing cresyl blue (in form of salt or in form of free base) with each successive drop of the same sample of sap taken for each one of the five measurements, where the maximum increase is a little over 0.1 pH. The observed pH value of the same sap remains constant throughout such a series of experiments when the electrode is washed with 0.1 N HCl and then with distilled water followed by final washing with test solution after each measurement. There is no danger of the acid diffusing out of the electrode which has been washed with acid into the sap, since the pH value of the sap containing no dye is found to be the same whether the electrode is first washed with the acid or not.

To avoid the errors arising from this source, the following procedure was adopted in the experiments described in the text. Before using the electrode it is removed from 0.1 N HCl, wiped with filter paper, washed in distilled water, and wiped again. Then the electrode is washed quickly in the solution, the potential of which is to be determined. After measurement, the electrode is washed in distilled water, wiped, and placed in 0.1 N HCl until the effect of the test solution on the electrode is completely removed. When the electrode is not in use, it is always kept in the 0.1 N HCl. The temperature of the solutions used to wash the electrode is kept within  $\pm 0.5^\circ\text{C}$ . of the temperature of the test solution.

*Time Curve for Glass Electrode Measurement.*—It is always necessary to continue measurement for about 5 or 10 minutes to see if there is any change in the potential with time. Such a change may occur from the effect of the solution on the electrode or from the change in the solution proper. The results show that there is no alteration in the pH value of the buffer solution at pH 6.65 within 5 minutes, nor in the pH value of the control sap. There is a tendency for the pH value of the sap containing the dye to rise with time, but the maximum rise in 5 minutes is only 0.05 pH, which is within an experimental error. The two measurements made within 2 minutes agree within  $\pm 0.03$  pH; the measurements given in the text represent the average of two readings made, (a) one-half minute after the electrode has first come in contact with the test solution, and (b) one minute and a half later.

*The pH Value of the Control Sap or the Sap Mixture.* If the pH value of the control sap changed rapidly after extraction, it would interfere with such experiments as are described in the text, but fortunately there was no alteration in the pH value of the sap nor of the sap mixture even on standing in a pyrex glass vial (stoppered) 2 hours after extraction. Even on keeping the sap in the refrigerator for 20 hours the pH value of the sap was found to increase only 0.2 pH.

Furthermore there was practically no alteration in the pH value of the sap nor of the sap mixture during the 15 minutes required for extraction. This test was made colorimetrically,<sup>18</sup> since by this method only a small capillary tube of sap was sufficient so that it was possible to complete the first determination only 3 minutes after the extraction had begun. The indicator method was found to be adequate since the pH value of the sap determined by this method agreed closely enough (within  $\pm 0.1$  pH) with the pH value determined by the glass electrode.

From day to day there was some variation in the pH value of the control sap, the maximum variation being about  $\pm 0.2$  pH. Owing to this an alteration in the pH value of the sap less than 0.2 pH should not be taken seriously. Though the pH value of the sap did not alter on standing for several hours, the measurement with the glass electrode was always made immediately after the sap was extracted.

Each reading given in the text is an average of 10 to 15 experiments employing altogether 400 cells or more.

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### III

#### *Nitella flexilis* (A)

*The Sap.*—The average sap of the control cells (taken from tap water) is at about pH 5.36 (Table I).

When the cresyl blue salt at a concentration in the neighborhood of 0.07 per cent is dissolved in the sap *in vitro* the pH value of the sap

<sup>18</sup> For method see Irwin, M., *J. Gen. Physiol.*, 1925-26, 9, 240, Section III.

is found to decrease by about 0.07 pH, which is too small an alteration to be considered seriously (Table I).

When about 0.07 per cent free base<sup>19</sup> of cresyl blue is dissolved in the sap *in vitro*, the pH value is found to increase by about 0.55 (Table I).

TABLE I

*pH Value of the Sap of Living Cells of Nitella flexilis (A)*

In presence of cresyl blue. Average temperature 21°C. Electrode I was used. Unless otherwise stated, penetration experiments were carried out with one end of the cell outside the solution in moist chamber, to avoid contamination of the sap. The duration of the experiments was about 15 minutes.

|  | Conc. of dye in sap in per cent | pH of sap | Increase in pH of sap over the control sap | Conc. of dye in sap containing protoplasm in per cent | pH of sap containing protoplasm | Increase in pH of sap containing protoplasm over the control sap containing protoplasm |
|--|---------------------------------|-----------|--|---|---------------------------------|--|
| Control cells in tap water.....  | 0                               | 5.36      |  | 0   | 5.97                            |  |
| Dye salt added <i>in vitro</i> .....   | 0.069                           | 5.29      |  |   |                                 |  |
| Free base of dye added <i>in vitro</i> .....   | 0.071                           | 5.91      | 0.55                                       | 0.070   | 6.48                            | 0.51   |
| Penetration from 0.002 per cent dye at pH 9.2.....                                     | 0.073                           | 5.86      | 0.50                                       | 0.065   | 6.45                            | 0.48   |
| Penetration from 0.025 per cent dye at pH 6.8.....                                     | 0.071                           | 5.87      | 0.51                                       | 0.074   | 6.37                            | 0.40   |
| Penetration from 0.004 per cent dye at pH 9.2.....                                     | 0.18                            | 6.66      | 1.30                                       |   |                                 |  |
| Free base added <i>in vitro</i> .....  | 0.16                            | 6.50      | 1.24                                       |   |                                 |  |
| Penetration from 0.002 per cent dye at pH 9.2, when cells are completely immersed..... | 0.070                           | 5.88      | 0.52                                       |   |                                 |  |
| After 8 hours in tap water containing no dye.....                                      | 0                               | 5.40      |  | 0   | 6.00                            |  |
| After 1 hour in buffer solution at pH 9.2, containing no dye.....                      | 0                               | 5.37      |  | 0   | 5.94                            |  |

These results prove that the two forms of the dye are distinguishable by determining the pH value with the glass electrode, the dye salt having very little effect on the sap, the free base of the dye distinctly raising the pH value of the sap. If therefore the same amount of dye penetrating the vacuole raises the pH value to about the same extent

<sup>19</sup> For method see Section II in the text.

as the free base of the dye, we may conclude that the dye penetrates as free base and not as salt. The following results show this to be the case.

When cells are placed<sup>19</sup> for about 15 minutes in 0.002 per cent dye solution at pH 9.2 until about 0.07 per cent dye has accumulated in the vacuole, the pH value of the sap is found to increase about 0.5 over that of the control sap (Table I). This increase<sup>20</sup> agrees closely with the increase obtained by dissolving 0.07 per cent free base of the dye in the sap (Table I), thus indicating that the dye penetrates chiefly as free base and not as salt.

Previous experiments<sup>21</sup> have shown that at pH 9.2 cresyl blue exists predominantly as free base so that it would not be surprising if the dye penetrated as free base from this solution. But at a lower pH value, where the dye salt predominates, does the dye enter as salt or as free base? To test this point the dye was allowed to penetrate for about 15 minutes from 0.025 per cent cresyl blue solution at pH 6.8 (in which there was a preponderance of the dye salt).<sup>21</sup> As shown in Table I, 0.07 per cent dye penetrating from this solution has brought about approximately the same increase (about 0.51 pH) in the pH value as 0.07 per cent dye penetrating from the solution at pH 9.2. These results prove that irrespective of the ratio of the free base to salt of the dye in the external solution, the dye penetrates as free base and not as salt.

The dye was furthermore allowed to penetrate to a still higher concentration (0.18 per cent) from 0.004 per cent cresyl blue solution at pH 9.2 in about 15 minutes, to see if further increase in the pH value of the sap would occur with a higher concentration of the dye in the sap. An increase<sup>20</sup> of about 1.30 pH over the control was found to take place, which agreed closely with the rise in the pH value by dissolving 0.16 per cent free base of cresyl blue in the sap *in vitro* (Table I).

The pH value of the sap in the presence of a lower concentration of

<sup>20</sup> These results prove that the alteration in the rate of entrance and exit of the dye formerly reported to occur in the cell after penetration of dye are not due to the production of acid in the sap to the extent of lowering the pH value of the sap below the normal. Irwin, M., *J. Gen. Physiol.*, 1925-28, 8, 147; *Proc. Soc. Exp. Biol. and Med.*, 1926-27, 24, 247.

<sup>21</sup> For reference see footnote 3.

cresyl blue (below 0.07 per cent) was determined but owing to the variation in the pH value of the control sap (to the extent of 0.2 pH) it is not desirable to draw conclusions from the lower concentration of the dye in the sap which brought about this increase in the pH value of the sap.

Owing to the fact that these experiments were carried out when a portion of the cell was exposed to the air in a moist chamber (to avoid contamination of the sap from the stained cell wall at the time of extraction) the question may be raised whether the increase in the pH value of the sap may not be caused by the penetration of the alkaline buffer as a result of injury to the cells under these experimental conditions. This "partial immersion" method, however, is proved to cause no injury in the duration of these experiments, as shown by the following experiments.

When the experiments were repeated by completely immersing the cells in 0.002 per cent dye at pH 9.2 for a little less than 15 minutes until 0.07 per cent dye had collected in the sap, the pH value of the sap was found to increase about 0.52 pH, which agrees closely with the rise brought about when cells were only partly immersed in the solution as previously stated (Table I). In this dye solution at pH 9.2 so little staining of the cell wall occurs (with this length of exposure) that no question of errors arising from contamination of the cell sap from the stained cell wall enters in the case of the cells completely immersed in this solution. If the partial immersion method causes injury, thereby hastening the penetration of the alkaline borate buffer, the rise in the pH value of the sap may be expected to be greater when this method is used than the rise when the cells are completely immersed. Since the rise in the pH value is about the same in both cases, we may conclude that the alteration in the pH value of the sap is not due to injury caused by the partial immersion method.

Furthermore the pH value of the cells which were partially immersed in this way in tap water for 8 hours or in the buffer solution at pH 9.2 (containing no dye) for 1 hour was the same as that of the control sap, thus showing that the partial immersion method does not injure the cells (for the duration of exposure described in the text) enough to affect the pH value of the sap (Table I).

*Sap Containing Protoplasm.*—The most striking fact is that after the

sap has been allowed to flow out without pressure, if the cell wall is squeezed so as to press the protoplasm into the sap, the pH value of this mixture is found to be about 0.6 pH higher than the sap proper (Table I). This may be chiefly due to the presence of the protoplasm, the pH value of which may be higher than that of the sap. Another possible explanation is that the fluid<sup>22</sup> which may exist in the space between the protoplasmic layer and the cell wall may also be squeezed into the sap and may help to raise the pH value of the sap.

In presence of 0.07 per cent cresyl blue free base the pH value of this mixture is increased about 0.51 over that of the control mixture (Table I). The cresyl blue penetrating from the dye solution at pH 9.2 or at pH 6.8 also raises the pH value of sap containing protoplasm by about 0.4 to 0.5 pH (Table I), thus showing that the dye penetrates chiefly as free base into this mixture. Undoubtedly not all the protoplasm can be squeezed into the sap and the protoplasmic layer is very thin so that in the mixture the sap preponderates over the protoplasm. For this reason it would be difficult to conclude from these results whether the dye penetrates chiefly as free base into the protoplasm as it does into the vacuole.

#### IV

##### *Nitella flexilis* (B)

To determine if the penetration of cresyl blue as free base will occur with another sample of *Nitella* obtained in a different locality, the experiments were repeated with cells collected elsewhere which are distinguished for convenience from *Nitella* (A) by the term *Nitella* (B).

*The Sap.*—The average pH value of the sap of *Nitella* (B) is pH 5.6 (Table II), which is about 0.2 pH higher than the pH value of the sap of *Nitella* (A).

On dissolving the 0.086 per cent free base<sup>19</sup> of cresyl blue in this sap *in vitro* the pH value of the sap is found to increase about 0.37 pH (Table II). This increase is about 0.2 pH less than is the case with *Nitella* (A). This may mean that the sap of *Nitella* (B) is more huffered than the sap of *Nitella* (A) (Tables I and II). On

<sup>22</sup> Irwin, M., *J. Gen. Physiol.*, 1926-27, 10, 75.



adding the dye salt to the sap there is practically no alteration in the pH value of the sap. When about 0.075 per cent dye penetrates the vacuole from the cresyl blue solution at pH 9.2 or 6.8, there is an increase in the pH value of 0.4 or 0.45 pH over that of the control sap (Table II), which agrees closely with the rise in the pH value when the free base of the dye is dissolved in the sap *in vitro*. In this case

TABLE II

*pH Values of the Sap of Living Cells of Nitella flexilis (B)*

In presence of cresyl blue. Average temperature at 22°C. Electrode I was used unless otherwise stated. The penetration experiments lasted about 15 minutes (with precautions to avoid contamination of the sap).

|   | Conc. of dye in sap in per cent | pH of sap | Increase in pH of sap over the control sap | Conc. of dye containing protoplasm in per cent | pH of sap containing protoplasm | Increase in pH of sap containing protoplasm over the control sap containing protoplasm |
|---|---------------------------------|-----------|--|--|---------------------------------|--|
| Control.....  | 0                               | 5.60      |  | 0  | 6.11                            |  |
| Free base of dye added <i>in vitro</i> .....  | 0.086                           | 5.97      | 0.37                                       | 0.077  | 6.43                            | 0.32   |
| Dye salt added <i>in vitro</i> .....  | 0.080                           | 5.55      |  | 0.085  | 6.07                            |  |
| Penetration from 0.002 per cent dye at pH 9.2.....  | 0.076                           | 6.00      | 0.40                                       | 0.065  | 6.35                            | 0.24   |
| Penetration from 0.025 per cent dye at pH 6.8.....  | 0.077                           | 6.05      | 0.45                                       | 0.069  | 6.36                            | 0.25   |
| Penetration from 0.01 per cent dye in tap water.....  | 0.076                           | 5.96      | 0.38                                       | 0.072  | 6.37                            | 0.26   |
| Cells placed for $\frac{1}{2}$ hour in buffer solution at pH 9.2 after cells had been in 0.002 per cent dye at pH 9.2 for about 15 minutes..... | 0.075                           | 5.95      | 0.35                                       | 0.070  | 6.31                            | 0.20   |
| Penetration from 0.002 per cent dye at pH 9.2 Electrode II.....   | 0.079                           | 5.98      | 0.38                                       | 0.070  | 6.39                            | 0.28   |
| Penetration from 0.002 per cent dye at pH 9.2 Electrode III.....  | 0.074                           | 5.95      | 0.35                                       | 0.068  | 6.36                            | 0.25   |

the increase in the pH value is a little less (about 0.1 to 0.2 pH) than that of the *Nitella (A)*.

Thus the results on *Nitella (B)* confirm those on *Nitella (A)*, showing that in both cases the dye penetrates the vacuole chiefly as free base.

*The Sap Containing Protoplasm.*—The pH value of the mixture of

the sap and the protoplasm extracted as before behaved very much like the mixture of *Nitella* (A) in that the pH value of the mixture is raised when the free base of the dye is dissolved in the mixture *in vitro* (Table II). On examining the pH values of the mixture after penetration of the dye they are found to be 0.1 to 0.2 pH lower than the corresponding pure sap containing approximately the same amount of dye (Table II). Such results may signify that some dye salt<sup>23</sup> penetrates into the protoplasm or that the pH value of the protoplasm is decreased when the dye penetrates as a result of production of acid by the protoplasm.

Though the results definitely show the penetration of cresyl blue into the sap to be chiefly as free base there is still one more uncertainty to be removed and this is in relation to the possibility of the rise in the pH value of the sap taking place as a result of penetration of an alkaline substance other than the dye owing to the injury caused by the penetration of the dye. Such a possibility is imminent owing to previous<sup>20</sup> results showing changes in the rate of penetration from the normal as a result of penetration of over 0.006 per cent dye. Since the concentration of cresyl blue in the vacuole in the present case is high (about 0.07 per cent), it is necessary to prove that the increase in the pH value of the sap is not due to the penetration of an alkaline substance other than the dye into those cells the condition of which is altered from the normal by penetration of the dye.

Since in all the experiments the length of exposure and the concentration of the dye which has penetrated into the sap are approximately equal, if the increase in the pH value of the sap were due to the penetration of an alkaline substance other than the dye, there should be a greater increase in the pH value of the sap when cells are exposed to the dye solution at pH 9.2 than at pH 6.8 or in tap water at pH 7.7. But this increase is found to be about constant irrespective of the external pH values and the external alkaline buffer concentrations, provided the concentration of dye in the sap is approximately the same (Table II). These results therefore point to the fact that whatever

<sup>23</sup> Experiments on penetration of dye from methylene blue, described in the subsequent paper, show that such a lowering is not due to the penetration of the dye salt, so that in all probability the same is true with the cresyl blue. Irwin, M., *J. Gen. Physiol.*, 1930-31, 14, 19.

secondary changes that might occur in the cell they do not admit an alkaline substance other than the dye into the vacuoles of these cells.

Another proof is as follows. If the increase of 0.4 pH in the sap over the control sap is not due to the penetration of alkaline buffer as a result of secondary changes, but is due to the penetration of about 0.08 per cent cresyl blue free base from the dye solution at pH 9.2, then by transferring such cells from the dye solution into the buffer solution at the same pH value containing no dye there should be no progressive increase in the pH value of the sap. Such is found to be the case experimentally, as shown in Table II, when even after  $\frac{1}{2}$  hour exposure of these cells to the buffer solution at pH 9.2 there is no further increase in the pH value of the sap. But a further increase is found to occur if more dye penetrates the vacuole.

These experiments were made with one electrode (I) only: afterward check experiments were made with several electrodes, which gave about the same results as Electrode I. The results from two other electrodes are recorded in Table II.

The spectrophotometric measurements show that the dye which has penetrated from the cresyl blue solution into the sap or into the sap mixture is identical with the external dye solution as well as the cresyl blue free base dissolved in the sap *in vitro* in that the absorption curves are characteristic of cresyl blue.

## V

### CONCLUSION

From these results we may conclude that the cresyl blue penetrates chiefly as free base and upon coming in contact with the sap at the vacuolar surface it is at once partly converted into the dye salt. At equilibrium the concentration of free base in the sap is proportional to that in the external solution. Thus the rate of penetration of the dye in form of free base and the concentration of the total dye (free base and salt) in the sap at equilibrium are dependent on the dissociation constant of the dye. For this reason both the rate of penetration and the concentration of the dye in the sap at equilibrium are increased with a rise in the external pH value (which increases the concentration

of free base of the dye) or with a decrease in the pH value of the sap (which increases the conversion of the free base to the dye salt).

It is possible that some of the dye may be converted to something different from the dye salt after the dye has penetrated the vacuole. Previous experiments<sup>20</sup> have shown that as the concentration of the dye in the sap through penetration reached a certain point the rate of penetration progressively increased while that of the exit decreased from that of the normal. Such an alteration in the rate is not due to the excess production of acid in the sap as shown by the preceding results. It may be caused by some kind of change in the dye as the dye penetrates the sap. Such a dye may be either another tautomer of cresyl blue, or a compound formed between the cresyl blue and the sap, or another dye. To affect the rate, as already stated, the reaction producing this dye must be less reversible than that producing cresyl blue salt or this dye must have a lower rate of exit from the vacuole into the external solution than cresyl blue salt.

These results cannot be due to exchange of dye cations for hydrogen ions, as this would involve a lower rate of penetration since the surfaces are non-aqueous and the concentration of hydrogen ions in the sap is too low. This will be discussed in subsequent papers.

#### SUMMARY

Glass electrode measurements of the pH value of the sap of *Nilella* show that cresyl blue in form of free base penetrates the vacuoles and raises pH value of the sap to about the same degree as the free base of the dye added to the sap *in vitro*, while the dye salt dissolved in the sap does not alter its pH value. It is proved conclusively that the increase in the pH value of the sap is due only to the presence of the dye and not to some other alkaline substance.

Spectrophotometric measurements show that the dye which penetrates the vacuole is chiefly cresyl blue.

When the protoplasm is squeezed into the sap, the pH value of the sap is higher than that of the pure sap. Such a mixture behaves very much like the sap in respect to the dye.



# STUDIES ON PENETRATION OF DYES WITH GLASS ELECTRODE

## V. WHY DOES AZURE B PENETRATE MORE READILY THAN METHYLENE BLUE OR CRYSTAL VIOLET?

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### I

The results<sup>1</sup> from measurements made with the glass electrode and spectrophotometer show that brilliant cresyl blue penetrates as free base into the vacuoles of living cells of *Nitella*. The present paper describes similar experiments with azure B, methylene blue, and crystal violet.

Since the methods for the condition of cells, extraction of the sap, penetration experiments, extraction of free base of dyes, making up of dye solutions, and measurements by means of the glass electrode have been published<sup>1</sup> in detail, they are omitted here. The dyes were obtained as salts. The azure B (in pure form) was made by W. C. Holmes of the Color Laboratory, Washington, D. C., methylene blue (medicinal) by Merck and Co., crystal violet by Grüber (obtained before 1914). These dyes affected the electrode slightly more than cresyl blue (causing the pH value of the sap containing the dye to increase between 0.1 and 0.2 pH value after the fifth measurement (when the electrode was not washed with acid), but these effects were entirely eliminated by washing the electrode with acid as already described.<sup>1</sup> Just as with cresyl blue, the readings did not vary within 5 minutes after the electrode was brought in contact with any one of these dyes.

<sup>1</sup> Irwin, M., *J. Gen. Physiol.*, 1930-31, 14, 1.

## II

*Azure B or Trimethyl Thionin**A. Nitella (A)*

*The Sap.*<sup>1</sup>—When about 0.07 per cent azure B salt is dissolved in the sap *in vitro* the pH value remains practically the same as that of the control (Table I), but when the same concentration of the dye in the form of free<sup>1</sup> base is dissolved in the sap *in vitro* the pH value is found to be about 1 pH above that of the control sap (Table I). Owing to this difference it is possible to find out which form enters the vacuoles of living cells of *Nitella* by determining the pH value of the sap after penetration.

When cells are placed in 0.01 per cent azure B solution at pH 9.2 for about 15 minutes until 0.07 per cent dye has penetrated the vacuole, the pH value of the sap is found to increase about 1 pH above that of the control sap (Table I).

Since azure B which has penetrated the vacuole increases the pH value of the sap to about the same extent as the same concentration of the dye in form of free base added to the sap *in vitro*, while the dye salt dissolved in the sap *in vitro* does not alter the pH value of the sap, it would seem that the azure B penetrates chiefly as free base and not as salt.

But the experimental results supporting this conclusion are not so numerous as with cresyl blue.<sup>1</sup> There is a greater possibility of injury to cells exposed to azure B than to cresyl blue. Since the rate of penetration at pH 9.2 is much slower with azure B than with cresyl blue, it was necessary to use a higher concentration<sup>2</sup> of azure B in order to maintain a comparable length of exposure to both these solutions. Cells were dead in this azure B solution after 3 hours' exposure while these cells in the cresyl blue solution were not dead until after 6 hours' exposure. Since injury hastens the entrance into the vacuole of substances whose penetration under normal conditions is negligible it is

<sup>2</sup> A lower concentration of azure B at pH 9.2 (causing penetration of 0.03 per cent dye in 15 minutes) was employed in which a rise in the pH value of the sap was found to be at about 0.5 pH over that of the control, and in which cells lived somewhat longer than in the solution described in the text. But even in this case, the results were not so conclusive as with cresyl blue.

all the more necessary to ascertain whether the cells were injured to such an extent that alkaline substances other than the azure B penetrated. Unfortunately it was not possible to determine this point so

TABLE I

*pH Values of the Sap of Living Cells of Nitella flexilis. (A and B)*

In presence of azure B and methylene blue. Average temperature 22.5°C. Duration of experiments was about 15 minutes with azure B and about 80 minutes with methylene blue (precaution was taken to avoid contamination of the sap). Electrode I was used unless otherwise stated.

|   | Conc. of dye in sap in per cent | pH of sap | Increase in pH of sap over the control sap | Conc. of dye in sap containing protoplasm in per cent | pH of sap containing protoplasm | Increase in pH of sap containing protoplasm over the control sap containing protoplasm |
|---|---------------------------------|-----------|--|---|---------------------------------|--|
| <i>Nitella A, azure B</i>                                       |                                 |           |  |   |                                 |  |
| Control.....  | 0                               | 5.25      |  | 0   | 6.09                            |  |
| Dye salt added <i>in vitro</i> .....                            | 0.07                            | 5.27      | 0.02                                       | 0.070   | 6.14                            | 0.06   |
| Free base of dye added <i>in vitro</i> .....                    | 0.73                            | 6.37      | 1.10                                       | 0.080   | 7.20                            | 1.06   |
| Penetration from 0.01 per cent dye at pH 9.2.....               | 0.075                           | 6.39      | 1.14                                       | 0.070   | 6.73                            | 0.64   |
| <i>Nitella B, azure B</i>                                       |                                 |           |  |   |                                 |  |
| Control.....  | 0                               | 5.52      |  |   | 6.00                            |  |
| Penetration from 0.01 per cent dye at pH 9.2.....               | 0.08                            | 6.52      | 1.0  | 0.080   | 6.50                            | 0.5  |
| Penetration from 0.01 per cent dye at pH 9.2. Electrode II..... | 0.08                            | 6.45      | 0.93                                       | 0.075   | 6.43                            | 0.43   |
| <i>Nitella A, methylene blue</i>                                |                                 |           |  |   |                                 |  |
| Control.....  | 0                               | 5.30      |  | 0   | 6.10                            |  |
| Dye salt added <i>in vitro</i> .....                            | 0.07                            | 5.28      |  | 0.075   | 6.17                            | 0.07   |
| *Free base added <i>in vitro</i> .....                          | 0.067                           | 6.31      | 1.01                                       | 0.065   | 6.99                            | 0.89   |
| Penetration from 0.04 per cent dye at pH 9.2.....               | 0.07                            | 6.43      | 1.13                                       | 0.073   | 6.78                            | 0.61   |

\* This free base is azure B extracted from methylene blue solution at pH 9.2.

satisfactorily as with cresyl blue. Owing to the heavy staining of the cell wall when cells were placed in the azure B solution (at all pH values) the dye at once began to diffuse into the vacuoles from the



cell wall when cells were transferred from the dye solution at pH 9.2, for example, into the buffer solution at the same pH value containing no dye so that it was not possible to prove whether or not the pH value of the sap would continue to increase without further penetration of the dye. Furthermore it was not possible to determine the penetration of azure B from tap water in the given period without employing a very high concentration of dye (too high for experiments without danger of causing injury to cells).

Thus the proof of the penetration of the dye in form of free base was less conclusive than with cresyl blue, but it seems probable that the azure B also penetrates as free base.

*Sap Mixture*<sup>1</sup> (*Sap Containing Protoplasm*).—Addition of 0.07 per cent dye salt or the free base to the mixture of sap and protoplasm produces much the same effect as on sap in that the dye salt brings about practically no alteration in the pH value of the mixture while the free base of the dye increases the pH value about 1 pH over that of the control mixture (Table I). But when the azure B penetrates until 0.07 per cent dye has collected in the vacuole, the increase in the pH value of this mixture is found to be only about 0.6 pH instead of 1 pH as was the case with the sap. Judging from the results obtained with penetration of dye from methylene blue (described in following section) we may conclude that this difference may be due to the production of acid by the protoplasm as a result of penetration of dye, and not to the penetration of azure B salt into the protoplasm. It is not due to the greater buffer action of the protoplasm since the pH value is increased to about the same extent when the azure B free base is added *in vitro* to the sap or to the mixture of the sap and the protoplasm. As the protoplasm consists of only a very thin layer it is not possible to obtain it free from the sap so as to study its behavior.

### *B. Nitella (B)*

Approximately the same results were obtained when the experiments were repeated with *Nitella*<sup>1</sup> (*B*) (Table I).

On employing Electrode II, the same results were obtained (Table I).

Spectrophotometric measurements show that the dye which has penetrated the vacuole is like the free base dissolved in the sap *in vitro*, and the external dye solution employed in giving absorption

curves characteristic of azure B with primary absorption maxima at about 650  $m\mu$ .

### III

#### *Methylene Blue or Tetramethyl Thionin*

##### *Nitella (A)*

*The Sap.*<sup>1</sup>—The pH value of the sap after 0.07 per cent dye had penetrated from 0.04 per cent methylene blue solution at pH 9.2 in 80 minutes was found to be about 1 pH higher than the control (Table I), which agreed closely with the rise in the pH value brought about by dissolving the 0.07 per cent free base of azure B in the sap (Table I). The methylene blue chloride when dissolved in the sap brought about practically no change in the pH value of the sap (Table I).

There was a striking similarity in the behavior of the dye absorbed by the vacuole and by the chloroform. When chloroform was shaken up with the methylene blue solution at pH 9.2, the dye appeared red in the chloroform. After evaporation of the chloroform, the dye residue was dissolved in sufficient sap to make the concentration 0.07 per cent. The pH value of this sap was found to be again about 1 pH higher than that of the control sap (Table I).

On determining by means of the spectrophotometric measurements the nature of the dye which has penetrated the vacuole from this methylene blue solution until 0.07 per cent has collected in the sap, the absorption curves showed that the dye consisted more of azure B than methylene blue with primary absorption maxima at 653 to 655  $m\mu$ . The dye absorbed by the chloroform was also found to be chiefly azure B with a primary absorption maximum at 650  $m\mu$ .

These results therefore confirm those obtained previously by spectrophotometric analysis<sup>3,4</sup> and support the conclusion previously made that the azure B free base present as impurity in methylene blue solution penetrates the vacuole so much more rapidly than methylene blue salt that the dye which collects in the vacuole consists chiefly of azure B and not methylene blue.

<sup>3</sup> Irwin, M., *Proc. Soc. Exp. Biol. and Med.*, 1926-27, 24, 425; 1927-28, 25, 563; *J. Gen. Physiol.*, 1928-29, 12, 147, 407.

<sup>4</sup> Experiments on spectrophotometric measurements were repeated by Brooks, who found methylene blue to penetrate instead of azure B. Brooks, M. M., *Proc. Soc. Exp. Biol. and Med.*, 1928-29, 26, 290. *Protoplasma*, 1929, 7, 46.

As soon as cells are injured, however, the methylene blue penetrates more rapidly so as to shift the absorption curve of the dye in the sap from that of a mixture containing more azure B than methylene blue to that of a mixture containing more methylene blue than azure B. Since in the present instance where there is 0.07 per cent penetration of dye from methylene blue solution at pH 9.2 it is the azure B free base that still penetrates predominantly, the cells thus exposed cannot be injured to the extent of permitting the dye salts to penetrate freely although the cells are dead in the methylene blue solution within three hours' exposure.

Owing to this toxicity of methylene blue solution and to the necessity of longer exposure of these cells to the solution, the results are not so convincing as those obtained on cresyl blue, but the evidence is in favor of the conclusion that predominantly azure B in form of free base penetrates the vacuoles unless cells are injured.

"*Sap Mixture*"<sup>1</sup> (*Sap Containing Protoplasm*).—The same results as in the case of the sap were obtained when the methylene blue or the dye extracted by the chloroform from the methylene blue solution was dissolved in the sap mixture *in vitro* (Table I). But on penetration of the dye from the methylene blue solution at pH 9.2 into the vacuoles the pH value of the sap mixture was raised only 0.6 pH over that of the control sap mixture instead of 1 pH increase, as was the case of the sap. This difference is not due to the greater penetration of methylene blue into the protoplasm than into the sap, because the dye in the sap mixture gives the same absorption curve as the dye in the sap, with a primary absorption maximum at 653 to 655 m $\mu$  (characteristic of a mixture of azure B and methylene blue with preponderance of the former). In all probability it is due to the production of acid by the protoplasm as a result of the dye penetration.

#### IV

#### *Crystal Violet*

#### *Nitella* (A)

Crystal violet is more toxic than other dyes employed here and the rate of penetration at any pH value into the cells of *Nitella* is not sufficiently rapid to cause an adequate penetration of the dye before there is a danger of injury to cells. In 0.001 per cent dye solution, for ex-

ample, the cells are dead after 1 hour, while the penetration after 15 minutes is too small to show whether the dye increases the pH value. The rate of penetration is approximately the same at pH 9.2 as at pH 5.5. As the dye penetrates, a violet precipitate appears in the sap with very little of the dissolved dye. The penetration of the dye is in all probability mostly due to injury. Crystal violet is a basic dye and it is soluble in chloroform so that according to Overton's theory it should penetrate the cells rather rapidly. Since the penetration is found to be slow, it is of interest to find out more about the behavior of this dye toward the sap of *Nitella in vitro* as a basis for an explanation of why it does not penetrate the vacuole readily.

When 0.07 per cent crystal violet chloride was dissolved in the freshly extracted sap, the pH value was not higher than that of the control sap (about pH 5.3).

The dye was absorbed by chloroform from aqueous solution at pH 5.5 and pH 9.2 in about the same amount. The stained chloroform was freed from the aqueous solution, allowed to evaporate, and the colored residue was dissolved in the sap. This did not raise the pH value of the sap above that of the control sap (whether the dye thus dissolved was obtained by shaking the chloroform with crystal violet solution at pH 5.5 or pH 9.2).

The behavior of crystal violet in sap is therefore different from that of the free base of cresyl blue or of azure B in that with crystal violet there is no difference between the dye salt and the dye absorbed by the chloroform from the solution at pH 9.2, while with cresyl blue or azure B the dye salt does not alter the pH value of the sap while the dye absorbed by the chloroform from the dye solution at pH 9.2 increases it.

Spectrophotometric determination shows that the absorption curves characteristic of crystal violet are obtained with the dye in buffer solutions at pH 9.2 or at pH 5.5, or with the dye which was absorbed by the chloroform from these solutions.

#### V

#### *Theoretical Considerations*

Do these facts help us to understand why azure B enters the vacuoles of living cells of *Nitella* rapidly while methylene blue and crystal violet penetrate very slowly?

The results show that in aqueous solution azure B exists chiefly in two forms, the dye salt and free base, while methylene blue and crystal violet exist chiefly in one form. They also show that azure B penetrates chiefly as free base and not as salt. Since the free base of azure B predominates over the salt at higher pH values, the rate of penetration of the dye increases as the external pH value rises. The increase of free base over salt depends on the "apparent dissociation constant" of the dye.

As soon as the dye in form of free base passes through the protoplasm and comes in contact with the sap in the vacuole, the greater portion of it is converted into the dye salt. The extent of this conversion is dependent on the basicity of the dye, and on the constituents of the sap (such as the hydrogen ion, organic salts, and protein). With *Nitella* sap, the chief factor controlling the conversion is the pH value of the sap. Owing to the low pH value of the sap and to the apparent dissociation constant of azure B, the free base of the dye is converted to the salt to such an extent that only a very small proportion of the dye exists in the sap as free base. At equilibrium the concentration of free base of the azure B in the sap is proportional to that of the free base of the dye in the external solution, so that the higher the concentration of the free base outside, the higher is the concentration of the total dye (free base and salt) in the sap. This explains why at equilibrium the concentration of the total dye in the sap is found to be higher when the pH value of the external solution is raised or the concentration of the dye is increased.

Why is it that the free base of the dye penetrates more rapidly than the dye salt? In these cases the living cells of *Nitella* behave as if the rate of penetration of the dye into the vacuoles is controlled by three phases only, that is, the non-aqueous layer lying between the external aqueous phase and the internal aqueous sap in the vacuole. The rate of penetration is partly controlled by the partition coefficients of the dye at these two phase boundaries. Azure B free base, for example, diffuses into the non-aqueous layer readily because the partition coefficient  $K_1$  of this form of dye between the non-aqueous layer and the external solution is high. But no matter how high the  $K_1$  is, the dye cannot diffuse into the vacuole unless the partition coefficient  $K_2$  of the free base between the non-aqueous layer and the sap is

low or the free base is largely converted by the sap into another form, the  $K_i$  of which is low. With azure B, the free base is predominantly converted to the salt, the  $K_i$  of which is low, so as to promote a rapid penetration and accumulation of the dye in the sap. The rate of penetration of dye will be greatly reduced by increasing the pH value of the sap and so causing less transformation of free base to the salt.

The exit of the dye may also be accounted for on the same theoretical basis which explains why the rate of exit is hastened with a decrease in the external pH value.

Methylene blue does not penetrate the vacuole readily because it exists as salt even at a high pH value such as pH 9.2, and the partition coefficient  $K_i$  of the dye salt is low.

The reason crystal violet does not penetrate the vacuoles readily, though the partition coefficient  $K_o$  is high, is because the partition coefficient  $K_i$  of the dye is also high. The results indicate that crystal violet exists only in one form in the range of pH values available for living cells of *Nitella* (between pH 5 and pH 9.2), so that there is no such transformation of free base into the salt as shown by azure B. It is uncertain whether the crystal violet exists as salt or as free base. From the structure it appears to be a strongly basic dye, but owing to its solubility in lipid it may be a very weak base. In case it is a weak base, it is too weak to increase the pH value even of the distilled water.

Owing to the penetration being dependent (at least in part) on more than one partition coefficient, the theory was called the "multiple partition coefficient theory."<sup>6</sup> It is uncertain yet whether this non-aqueous layer is lipid in nature but its behavior is very much like that of lipid. This theory is not identical with Overton's even if the non-aqueous layer proves to be a lipid because Overton considered only one partition coefficient,  $K_o$ , between the non-aqueous layer and the external solution. His theory is tenable in so far as those dyes which are not soluble in lipid do not readily penetrate the vacuoles of these living cells, but it fails to explain why those dyes which are soluble in a lipid do not readily penetrate. In the case of crystal violet, for example, it becomes explainable only when the second par-

<sup>6</sup> For the theory of multiple partition coefficients see Irwin, M., *Proc. Soc. Exp. Biol. and Med.*, 1927-28, 25, 127; *J. Gen. Physiol.*, 1927-28, 11, 111; 1928-29, 12, 147, 407.

tition coefficient,  $K_1$ , is considered. Furthermore, the accumulation of the dye in the vacuole cannot be explained on the basis of his theory.

Such instances show the simplest type of penetration. In more complicated cases we must consider other partition coefficients. In view of the fact that a living cell of *Nitella* consists of a heterogeneous system composed of an outer non-aqueous layer (in contact with the external solution), the middle aqueous layer of the protoplasm, the inner, non-aqueous layer (in contact with the sap in the vacuole), and the aqueous sap, the rate of penetration may be controlled by two or more of the four partition coefficients. The discussion of these cases will be deferred to a later publication.

#### SUMMARY

Glass electrode measurements of the pH value of the sap of cells of *Nitella* show that azure B in the form of free base penetrates the vacuoles and raises the pH value of the sap to about the same degree as the free base of the dye added to the sap *in vitro*, but the dye salt dissolved in the sap does not alter the pH value of the sap. It is concluded that the dye penetrates the vacuoles chiefly in the form of free base and not as salt.

The dye from methylene blue solution containing azure B free base as impurity penetrates and accumulates in the vacuole. This dye must be azure B in the form of free base, since it raises the pH value of the sap to about the same extent as the free base of azure B dissolved in the sap *in vitro*. The dye absorbed by the chloroform from methylene blue solution behaves like the dye penetrating the vacuole. These results confirm those of spectrophotometric analysis previously published.

Crystal violet exists only in one form between pH 5 and pH 9.2, and does not alter the pH value of the sap at the concentrations used. It does not penetrate readily unless cells are injured.

A theory of "multiple partition coefficients" is described which explains the mechanism of the behavior of living cells to these dyes.

When the protoplasm is squeezed into the sap, the pH value of the mixture is higher than that of the pure sap. The behavior of such a mixture to the dye is very much like that of the sap except that with azure B and methylene blue the rise in the pH value of such a mixture

is not so pronounced as with sap when the dye penetrates into the vacuoles.

Spectrophotometric measurements show that the dye which penetrates from methylene blue solution has a primary absorption maximum at 653 to 655  $m\mu$  (i.e., is a mixture of azure B and methylene blue, with preponderance of azure B) whether we take the sap alone or the sap plus protoplasm.

These results confirm those previously obtained with spectrophotometric measurements.





# A STUDY OF THE BACTERICIDAL ACTION OF ULTRA VIOLET LIGHT

## III. THE ABSORPTION OF ULTRA VIOLET LIGHT BY BACTERIA

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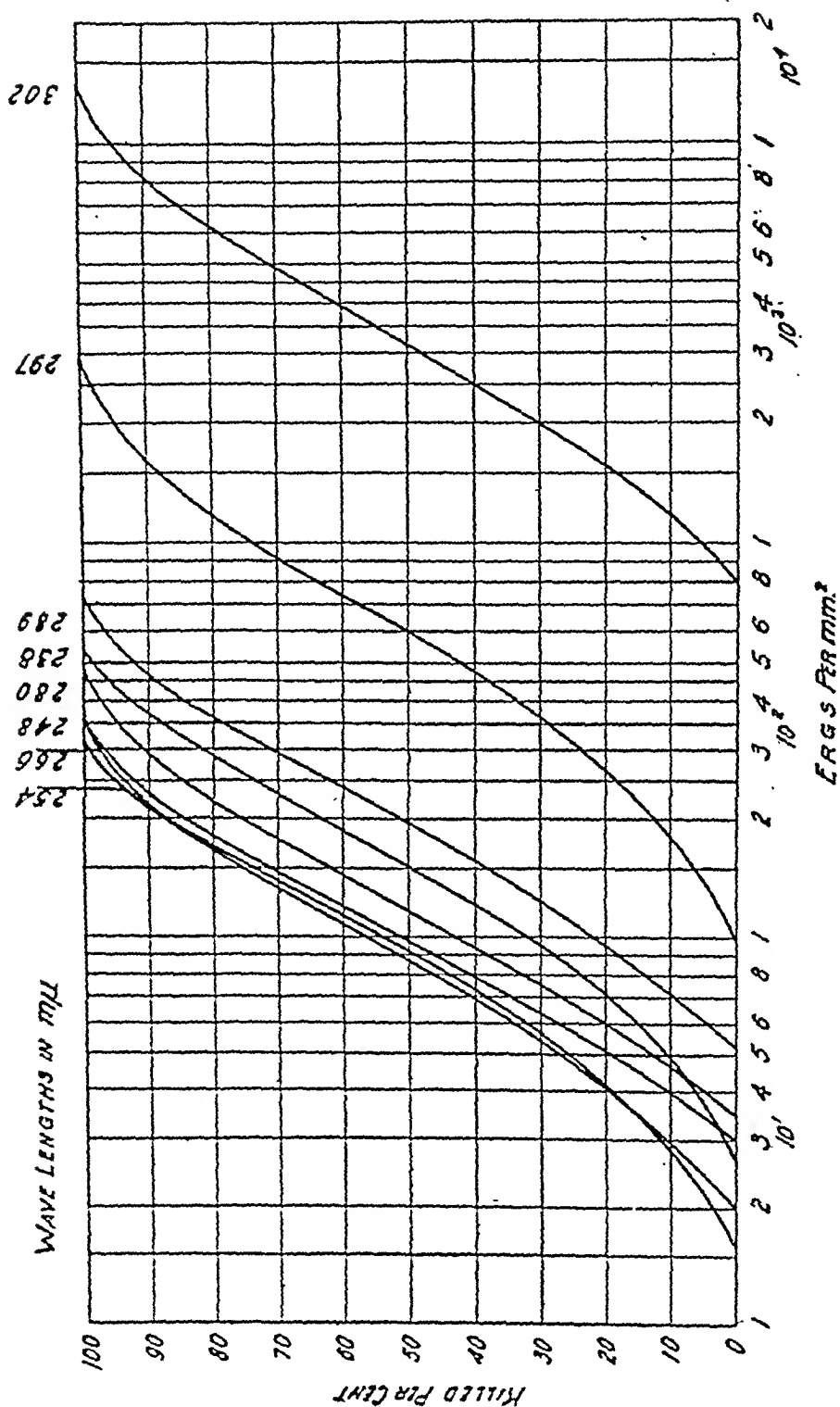
In this study of the bactericidal action of ultra violet light the first paper (1) described the reaction of an 18 hour culture of *Staphylococcus aureus* to monochromatic radiations. It was shown that the course of the reaction among large numbers of organisms was approximately the same at each wave length studied but that widely different incident energies were required at different wave lengths to produce these similar effects.

The second paper (2) discussed the limits of the bactericidal zone, showed that the reaction had a low temperature coefficient, (approximately 1.1), gave evidence that within the variations of the methods used no significant errors were introduced by differences in the measured intensity of the source or in the hydrogen ion concentration of the medium, and indicated that plane polarization of the incident light had no effect upon the reaction.

The present paper deals with the absorption of ultra violet light by intact bacteria. A final paper in this series will discuss structural and chemical units of bacterial protoplasm that may prove to be involved in the reaction which results in the organism's death.

### *Incident Energy Relationships at Various Wave Lengths*

Text-fig. 1, reproduced from the first paper of this series, shows that although the course of the bactericidal reaction was approximately the same at each wave length studied, these similar curves were found at very different incident energy levels at different points in the ultra violet spectrum.



TEXT-FIG. 1. Incident energies required for bactericidal action at various wave lengths in the ultra violet spectrum

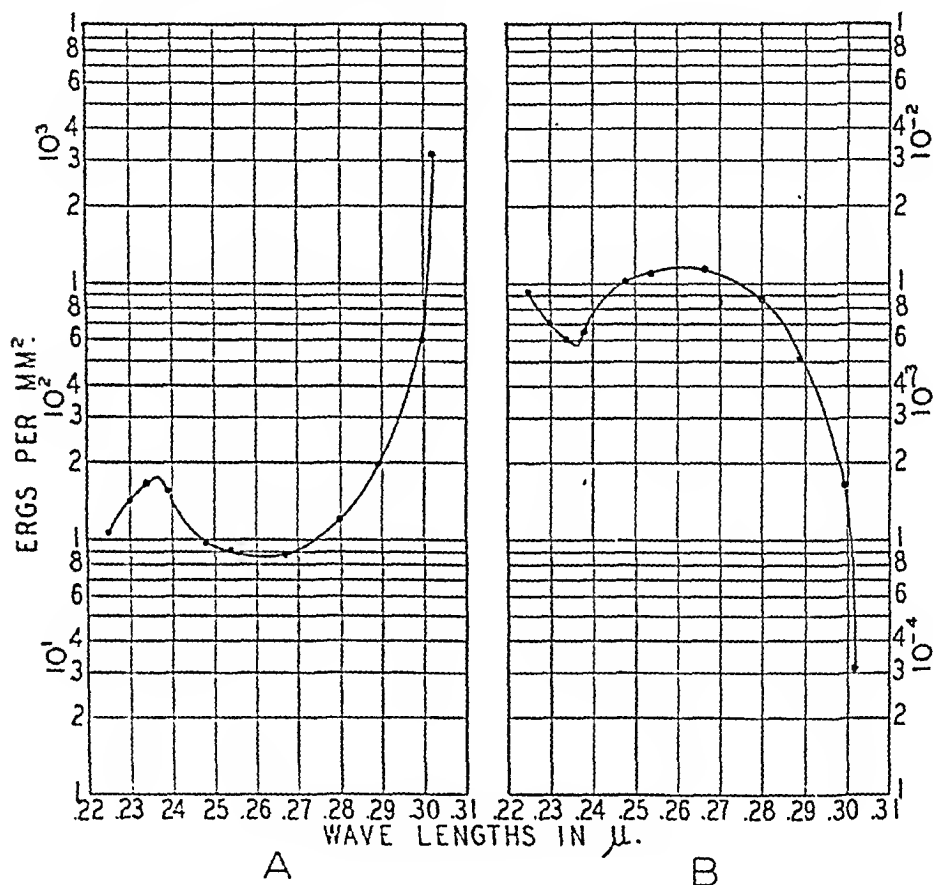
For example, if the incident energy required to kill half the exposed staphylococci be taken as an index, this energy requirement ranged from 3,150 ergs per mm.<sup>2</sup> at 302 m. $\mu$ , a wave length near the limit of bactericidal action, to 88 ergs at 266 m. $\mu$ . At other wave lengths, either above or below 266 m. $\mu$ , more incident energy was required. The destruction of 50 per cent of the microorganisms is chosen as the index because it is in the most accurately determined part of the curves where the mortality rate is least affected by variations in individual resistance.

If the incident energies involved in 50 per cent destruction be plotted and joined by a continuous line the resulting curve appears as in Text-fig. 2A. Parallel experiments on an 18 hour culture of *Bacillus coli* gave bactericidal energy curves similar in trend to those for *S. aureus*, and although complete statistics were not obtained on this bacterium the middle or exponential portion of the lethal reaction curves was determined by repeated observations at each wave length studied. Text-fig. 3A shows the incident energies involved in the destruction of 50 per cent of the exposed *coli* organisms. Its essential similarity to the corresponding *aureus* curve is apparent. Both curves would probably be somewhat modified in detail if more wave lengths were available for study.

These characteristic curves (Text-figs. 2A and 3A) show clearly that less incident energy is required between 260 and 270 m. $\mu$  than in any other region of the bactericidal zone examined, and point toward a second minimum below 230 m. $\mu$ . The presence of a sharp peak in the energy requirement near 240 m. $\mu$  appears to be equally significant. Due apparently to the use of but a few wave lengths in the bactericidal zone, or to failure to measure spectral intensities, or to crude methods of estimating bacterial destruction, the occurrence of a minimum at about 266 m. $\mu$ , and of the peak in the curve near 240 m. $\mu$  has been overlooked by most investigators. Usually they have been content with the conclusion that the shorter the wave length the more marked the bactericidal action (3).

Bang (4) was apparently the first to observe regions of special bactericidal "effectiveness" or of corresponding bacterial susceptibility. Using a 30 ampere carbon arc through a spectrograph at 20 m. $\mu$  intervals, and estimating bactericidal efficiency mainly by relative exposure, he found two regions of maximal action, an

"inner maximum" between 340 and 360 m. $\mu$ , and an "outer maximum" between 240 and 260 m. $\mu$ . The lethal exposure varied from 1920 seconds at 330 to 300 m. $\mu$  through 120 seconds at 300 to 280 m. $\mu$  to 4 seconds at 280 to 260 m. $\mu$  and 2 seconds at 260 to 240 m. $\mu$ . Then longer exposures were required. The zone from 240 to 220 m. $\mu$  needed 20 seconds, that between 220 and 210 m. $\mu$ , 30 seconds, and that between 210 and 200 m. $\mu$  required 120 seconds exposure to kill the organisms.



TEXT-FIG. 2. A. Curve of incident energies involved in the destruction of 50 per cent of *S. aureus*.

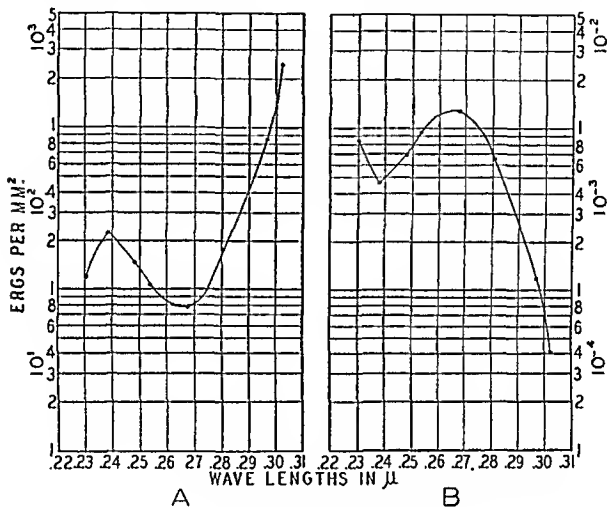
B. Curve of the reciprocals of 2A.

These longer exposures at short wave lengths were evidently due to the rapid decrease in intensity of the source employed. Mme. Henri (3) thought that both of Bang's maxima should be ascribed to variations in intensity of his carbon arc.

Newcomer (5) exposed *B. typhosus* in quartz capillary tubes to narrow bands of the iron arc spectrum and counted surviving bacteria plated in agar, after exposures of 5 or 10 minutes. His figures, like Bang's, indicate a region of maximum effect

between 254 and 268 m. $\mu$ , but his iron arc varied so much in spectral intensity that he did not find any significance in this peak of effectiveness. He concluded that "equal intensities produce equal effects in the regions 2100-2800. If there is a maximum in this region it is at most only slight and would be in the neighborhood of 2600."

When Mashimo (6) varied the exposure of bacteria in a spectrograph from 15 seconds to 80 minutes he found the first evidence of bactericidal action at 275 m. $\mu$ .



TEXT-FIG. 3. A. Curve of incident energies involved in the destruction of 50 per cent of *B. coli*.

B. Curve of the reciprocals of 3A.

With somewhat longer exposures the zone widened rapidly so that with a 3 minute exposure it extended from below 210 to above 280 m. $\mu$ . The marked action at 275 m. $\mu$  was evidently due to a relatively high intensity of his source at this wave length, as an examination of his published photograph shows.

Thus variations in spectral intensity, with no adequate methods of measurement or control, made it difficult to interpret the maxima found by Bang, Newcomer, and Mashimo, and these authors laid no stress upon them.

In the present study the measurement of the monochromatic radiant energy in absolute units focuses attention upon the marked differences in the incident energies required at different wave lengths to kill *S. aureus* and *B. coli*. The shape of the energy curves for 50 per cent destruction immediately suggests that it is the specific absorption of the ultra violet radiations that gives the curves significance. This relation of incident energy to its specific absorption is made the more striking by plotting the reciprocals of these energies (Text-figs. 2B and 3B), by which graphs not unlike absorption curves, with maxima at 266 and beyond 230 m. $\mu$  are produced.

As a first step in the further analysis of the bactericidal reaction it is obviously necessary to compare these curves with those for the absorption of ultra violet energy by the corresponding bacteria.

### *The Absorption of Ultra Violet Light by Bacteria*

Apparently studies of the absorption spectra of bacteria have been confined hitherto to bacteria in suspension in a fluid medium (7, 8). Suspensions in liquids are unsuitable for such examinations. Reflection and refraction from the bacterial bodies, with consequent scattering of light so that bacterial suspensions are opaque even in the visible region of the spectrum, and the difficulty of estimating the number of organisms traversed, frustrate any attempt to obtain results of quantitative significance.

But a loopful of bacteria may be taken *en masse* from the surface of an agar slant and pressed between quartz plates into a layer so thin that it is all but colorless in visible light, and so transparent that objects may be seen through it clearly and without distortion. The bacteria are in optical contact and form a homogeneous medium for the transmission of visible or ultra violet light. Such a film is composed almost entirely of bacterial cells and the immediate products of their metabolism. Tests show that films of like thickness of the agar medium from which the bacteria were removed absorb no significant amounts of ultra violet light.

Such a film of bacteria may be set up in one optical path of a quartz photometer, with similar plates of quartz and a drop of glycerol in the other path as a control, and the absorption coefficient of the organisms readily obtained in the manner commonly employed for chemical solutions (9).

Two beams of light from the same source, which have passed through the specimen under examination or its control in the photometer, are spread by a quartz spectrograph into parallel spectra in the plane of a photographic plate. The energy that traverses the control path is subject to quantitative variation by means of a sector shutter, and with the shutter set at predetermined openings a series of photographs is made. Then the point, or points, of equal blackening in each pair of spectral photographs indicate the wave lengths at which the test specimen and the sector shutter in the control path have reduced the original intensity of the light to the same degree.\*

In such experiments it is necessary to know accurately the depth of the medium traversed in order to calculate the coefficients of absorption for a layer of unit thickness. The standard or unit of thickness in these observations was chosen as  $0.8\ \mu$ , the average diameter of *S. aureus* (10), so that the coefficients of absorption were obtained for a single layer of bacteria. The shape of *B. coli* and its wide variations in size precluded even so crude an estimate of a "single layer," so the coefficients for *B. coli* were figured arbitrarily for a layer of the same thickness ( $0.8\ \mu$ ) to permit a comparison with the results for *S. aureus*.

The thickness of these films of bacteria between quartz plates was found to lie between 5 and  $15\ \mu$ . Since the method of measurement employed (11) is easy, and is accurate (in mieroins) to the second or third decimal place it may be described in brief. The method is based on interferometry, namely, the measurement of the interference bands formed by the coincident spectra of white light reflected into a wavelength spectroscopel from the two quartz surfaces enclosing the bacterial film.

A point source of white light (carbon arc, concentrated tungsten filament, or even a flashlight bulb with the filament vertical), is set up at a measured angle of incidence ( $60^\circ$ ) to the film specimen, so that its light is reflected from the back surface of the first quartz plate and from the front surface of the second (the surfaces enclosing the film), into a wavelength spectroscopel. The reflecting surfaces must be chosen near the bacterial film, but not to include it, since adequate reflection occurs only when air is the medium between the two plates. With a proper set-up, and films of suitable thickness a series of vertical interference bands will cut across the spectrum.

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\* Measurements of absorption by such films made in 1923 with thermocouple and galvanometer (*Proc. Soc. Exp. Biol. and Med.*, 1923, 21, 61), were evidently not as accurate as those obtained by the present method and have been discarded.



Then the distance ( $l$ ) between the two plates (the thickness of the included film) is found by noting the wave lengths ( $\lambda_1$  and  $\lambda_2$ ) between which any convenient number ( $n$ ) of interference bands is counted for substitution in the following equation:

$$l = \frac{n \lambda_1 \lambda_2}{2 \mu \cos r (\lambda_1 - \lambda_2)}$$

$\mu$  is the refractive index of the medium, and  $r$  is the angle of incidence and reflection. Since air is the medium  $\mu = 1$ , and with  $r$  at  $60^\circ$   $\cos r = 0.5$  so that the equation becomes

$$l = \frac{n \lambda_1 \lambda_2}{(\lambda_1 - \lambda_2)}$$

For example, with the apparatus set up as described 5 interference bands are counted between  $\lambda_1 = 7241 \text{ \AA u.}$  and  $\lambda_2 = 4638 \text{ \AA u.}$  It is only necessary to multiply these wave lengths together, divide by their difference, and multiply the result by 5 to determine that the distance between the plates *in the area examined* is  $6.45 \mu$  or  $0.00645 \text{ mm.}$

In these experiments the films of bacteria, pressed out by hand, were not strictly plane parallel, but often thicker at one edge than at the opposite one, so four readings were taken at  $90^\circ$  intervals around the rim of each film and combined for an average thickness. The differences in thickness in the small central area exposed in the photometer were not so great as to make it desirable to obtain a mean exponentially according to Lambert's law. For example:

Film 1. *B. coli*.  $90^\circ$  readings at edge of film: 5.58, 6.17, 5.63, and  $5.66 \mu$ . Average  $5.76 \mu$ .

Film 2. *S. aureus*.  $90^\circ$  readings at edge of film: 9.21, 11.66, 11.24, and  $10.53 \mu$ . Average  $10.66 \mu$ .

Given the average thickness of each film, the number of layers ( $n$ ) of bacteria it contained was then available for the determination of the coefficients of absorption for a layer  $0.8 \mu$  thick. For this purpose the familiar equation of Lambert's law was used, to which Wood (11) says that no exception has ever been found that was not attributable to experimental error. Thus if  $a$  is the fraction of the original intensity ( $I_0$ ) transmitted by each unit layer, the intensity ( $I_t$ ) observed after passage through  $n$  layers is given by the equation:

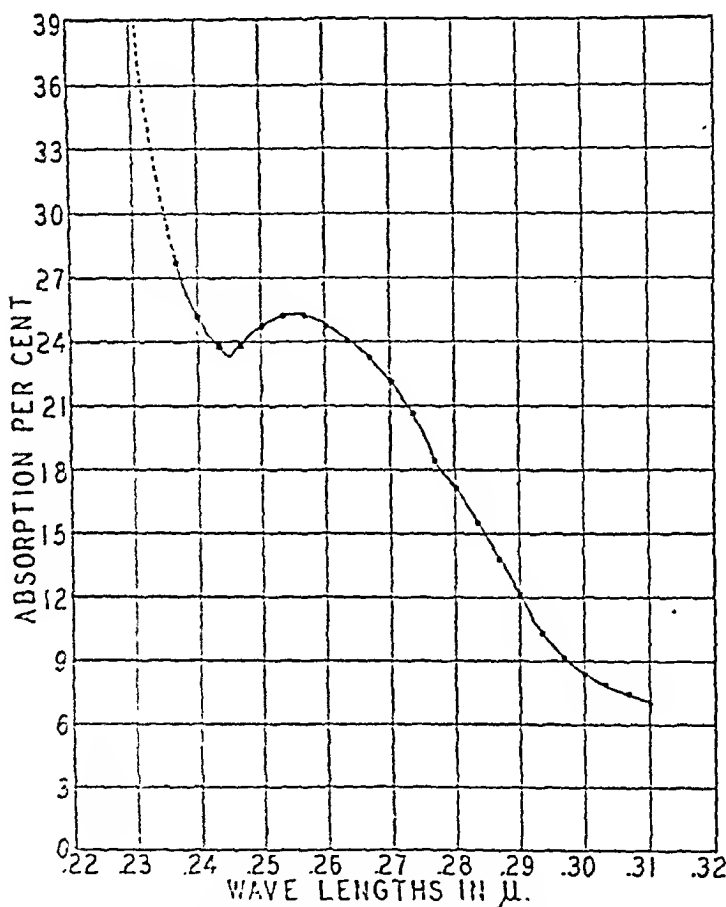
$$I_t = I_0 a^n$$

The relation of  $I_t$  to  $I_0$  is chosen at appropriate intervals on the sector shutter of the quartz photometer, as described above, and this equation solved for  $a$  gives the coefficient of transmission at the wave length where the two spectra match. Then  $1-a$  is the corresponding coefficient of absorption for a single layer of bacteria  $0.8 \mu$  thick.

The mean absorption curve from five series of determinations with *S. aureus* is given in Text-fig. 4, and from five series with *B. coli* in Text-fig. 5. The curves are characteristic, and similar to those found for various biological tissues and fluids containing proteins or protein derivatives (12, 13, 14) on which their main features evidently depend. Differences in the curves for the two organisms are apparent, but in view of the experimental errors involved in such determinations they probably should not be stressed.

The general similarity of these absorption curves to the reciprocals of the curves for bactericidal incident energy is obvious. All four curves rise rapidly from low levels beyond  $300 \text{ m.}\mu$  to a maximum between  $260$  and  $270 \text{ m.}\mu$ , then drop to a minimum near  $240 \text{ m.}\mu$  and rise again toward a limit beyond the range of experimental observation. One is tempted to correct the incident energies involved in the bactericidal reaction by these absorption coefficients for the entire organisms in order to obtain an approximation of the total energies absorbed. Yet the two sets of curves show important points of difference also, especially in the location of the dip near  $240 \text{ m.}\mu$ , and a closer consideration of their relationship indicates that such a quantitative correction would be futile. The sum of the absorption coefficients of all the chemical entities in the bacterial cell cannot be expected exactly to correct the wide differences found in the bactericidal incident energies at different wave lengths unless every chemical group is involved in the bactericidal reaction in exact proportion to its contribution to total absorption. Such a hypothesis is hardly tenable. It seems more probable that it is the effect of ultra violet energy on a single vital and sensitive structural or chemical unit that results in subsequent failure in cell multiplication. Rahn (15) has recently figured from the curves of abiotic reactions among large numbers of single cell organisms that death of the cell probably involves only a single chemical entity. As Coblentz and Fulton (16) have suggested, it is to be

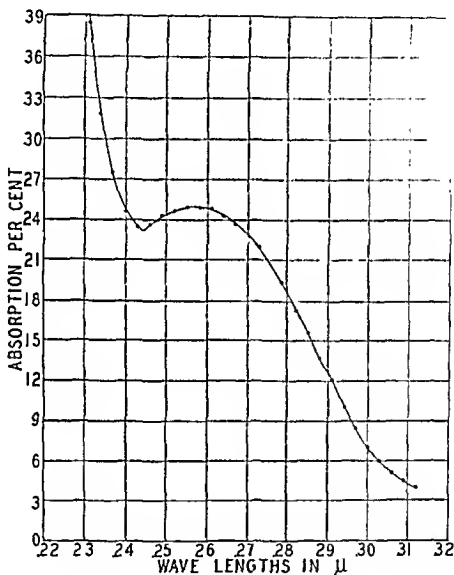
presumed that only a small fraction of the total absorbed energy first affects such an essential structure, and so leads to the death of the cell. Neither the reciprocals of the energy curves, which are undoubtedly modified by the absorption of light by elements not involved in the



TEXT-FIG. 4. The coefficients of absorption of ultra violet light by a layer of *S. aureus* 0.8  $\mu$  thick.

bactericidal reaction, nor the curves for total absorption give an accurate picture of the absorption curve of this vital substance, whatever it may be. Yet their similarities and their differences are alike useful in the further search for such an essential and sensitive element in the cell's structure and economy.

Thus it may be predicted that there are a number of chemical entities or aggregates in the living cell which have rather similar coefficients of ultra violet absorption, and that the sum of these similar absorption curves largely determines the shape of the curve for the entire cell.



TEXT-FIG. 5. The coefficients of absorption of ultra violet light by a layer of *B. coli*  $0.8 \mu$  thick.

Among these substances will be found the one essential element first affected by ultra violet light in the bactericidal reaction, and its absorption curve will be similar to, though not identical with, the reciprocal of the lethal energy curve. Finally it seems improbable that this sensitive substance is uniformly distributed throughout the cell's pro-

toplasm. An examination of the evidence for its concentration in the cell nucleus, and the further search for evidence of its chemical character are reserved for the final paper of this series.

#### SUMMARY

The simple conclusion of former investigators that the shorter the wave length of ultra violet light the greater the bactericidal action is in error. A study with measured monochromatic energy reveals a characteristic curve of bactericidal effectiveness with a striking maximum between 260 and 270 m. $\mu$ . The reciprocal of this abiotic energy curve suggests its close relation to specific light absorption by some single essential substance in the cell.

Methods are described for determining the absorption curve, or absorption coefficients, of intact bacteria. These curves for *S. aureus* and *B. coli* have important points of similarity and of difference with the reciprocals of the curves of bactericidal incident energy, and point the way in a further search for the specific substance, or substances, involved in the lethal reaction.

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# THE REACTIONS OF CYANIDE WITH GLOBIN HEMOCHROMOGEN

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## *Hemochromogen and the Cyanide Compounds of Reduced Heme*

Four pyrrol groups can combine to form a porphyrin. There are many different porphyrins with different side chains (Fischer, 1927). Each porphyrin can combine with iron to form a heme. The familiar hemin crystals are composed of the chloride of the particular iron-porphyrin or heme which is part of hemoglobin. Hemochromogen is defined as a pigment containing heme whose spectrum has the two characteristic bands first described by Stokes (1864).

In previous papers (Anson and Mirsky, 1925, 1928) it was shown that (1) Every hemochromogen consists of reduced heme joined to some nitrogen group; (2) Hemochromogen is always partly dissociated into and in equilibrium with its two components:



(3) Ordinarily, nitrogen substances react with reduced heme to form only one hemochromogen-like pigment. Cyanide, however, can react with reduced heme to form two different hemochromogen-like pigments; (4) The first cyanide compound of reduced heme contains one cyanide group per heme.

These results have been confirmed and evidence further given that the second cyanide compound contains two cyanide groups per heme (Hill, 1929).

The properties of cyanide are such as to permit of the estimation of the composition of the two cyanide compounds of reduced heme. But

it has not been clear what the relation is of these two compounds, both of which resemble hemochromogen spectroscopically, to an ordinary hemochromogen such as globin hemochromogen. The nitrogenous substances other than cyanide have the advantage of forming with heme only a single hemochromogen-like pigment which is undoubtedly a typical hemochromogen. But the properties of the other nitrogenous substances, as will be explained later in this paper, are such as to make difficult and uncertain the estimation of the compositions of the hemochromogens which they form from heme. As a result it has not been known whether hemochromogen in general contains one or two nitrogen groups per heme and it has not been known which of the two cyanide compounds is the typical hemochromogen, or why cyanide in particular forms two compounds with reduced heme. It was stated in the previous paper (Anson and Mirsky, 1928) that the relation of the cyanide compounds to ordinary hemochromogen was being studied by an investigation of the reactions of cyanide with heme in the presence of other nitrogenous substances.

The present experiments show that cyanide can react with globin hemochromogen in two entirely different ways. First, cyanide without displacing globin can combine with globin hemochromogen. In this reaction cyanide is *not* competing with globin for a place of attachment to the heme; it is behaving not like a typical nitrogenous hemochromogen-forming substance but probably like carbon monoxide. In addition, however, cyanide can displace globin from its combination with heme just as can ammonia and other nitrogenous substances (Anson and Mirsky, 1925, 1928). In the light of these results, the simplest although not the only explanation of the reactions of cyanide with heme in the absence of globin is that cyanide first combines with heme to form a typical hemochromogen containing one nitrogen group per heme and then combines with this cyan-hemochromogen just as it combines with globin hemochromogen.

### *The Biological Importance of the Hemochromogens*

The biological interest in the chemistry of hemochromogen used to consist solely in its relation to the chemistry of hemoglobin which was the only heme pigment of known physiological importance. It is now clear that hemochromogens as such are of wide distribution and peculiar importance in nature. Pigments related to hemochromogen are present in aerobic tissues of plants and animals

generally in concentrations great enough to permit of direct spectroscopic observation (MacMunn, 1886; Keilin, 1925, 1926; reviewed in Anson and Mirsky, 1930b). In addition the investigations of Warburg and his colleagues show that there is universally present in much smaller amount another substance related to heme and probably to hemochromogen which plays an essential rôle in the catalysis of biological oxidations (reviewed in Warburg and Negelein, 1929, and Anson and Mirsky, 1930h).

The study of the heme pigments in nature has depended entirely on a knowledge of the chemistry of pure known heme compounds *in vitro*. It is therefore important to know as much as possible about the kinds of reactions in which heme pigments can take part. In particular the ability of heme to react with nitrogen groups is significant. Aerobic cells contain a substance which can catalyze oxidations. The catalysis stops when this respiratory enzyme is combined with carbon monoxide and goes on again when the carbon monoxide is dissociated from its compound with the enzyme by light (Warburg, 1926). Similarly heme can catalyze oxidations *in vitro* and it forms with carbon monoxide a catalytically inactive compound which can be dissociated by light. But the catalytic activity of the heme so far tried is much less than that of the cellular enzyme and the sensitivity of the heme's carbon monoxide compound to light is also much less. When heme is combined with some nitrogenous hemochromogen-forming nitrogen group then the catalytic activity of heme and the sensitivity of its carbon monoxide compound to light are very much increased (Krebs, 1928). In other words, combination with a nitrogen group makes heme more like the cellular respiratory enzyme (reviewed in Warburg and Negelein, 1929, and Anson and Mirsky, 1930b).

### *The Reactions of Globin and Cyanide with Heme*

In the present experiments different amounts of cyanide are added to reduced heme in the presence of different concentrations of globin. From the spectra of the resulting solutions it can be decided when the cyanide is competing with globin for a place on the heme molecule and when the cyanide is not competing with globin. Before describing the experiments in detail it is necessary to recall what has already been shown to happen when either globin or cyanide alone is added to reduced heme.

If denatured globin is added gradually to an alkaline solution of reduced heme the spectrum of reduced heme is gradually and finally completely replaced by that of globin hemochromogen. The addition of further globin then causes no further change in the spectrum (Anson and Mirsky, 1928). If cyanide, however, is gradually added to reduced heme there appears first a typical hemochromogen spectrum whose  $\alpha$  band is 50 Å to the blue of that of globin hemochromogen.



But then on further addition of cyanide there appears a second two-banded spectrum similar to but definitely different from that of a typical hemochromogen. The  $\alpha$  band is 100 Å to the red of that of globin hemochromogen and is much weaker than the  $\alpha$  band of either globin hemochromogen or the first cyanide compound. This second cyanide compound obtained by adding an excess of cyanide has long been known (see spectrum in Oppenheimer's Handbuch, 1909). The first cyanide compound, which can exist only in a narrow range of low cyanide concentrations, was not observed until recently (Anson and Mirsky, 1928).

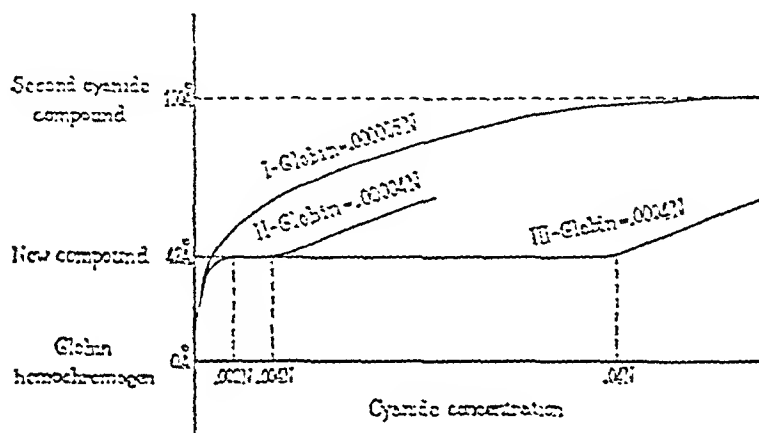


FIG. 1. Positions of  $\alpha$  bands of pigments obtained on adding cyanide to globin hemochromogen.

### *The Reactions of Cyanide with Globin Hemochromogen*

The new experiments are these:

1. To a dilute alkaline solution of globin hemochromogen prepared from hemoglobin (0.0084 per cent or 0.000005  $\times$  in respect to the iron of hemoglobin) there is added more and more cyanide. The  $\alpha$  band is shifted more and more to the red, until after a shift of 100 Å it has reached the position characteristic of the second cyanide compound of reduced heme prepared by adding cyanide to heme in the absence of globin. (See Curve 1. In order to give a simple picture of all the phenomena in one graph, the graph has been made schematic and grossly inaccurate. The actual observations are given in the tables.)

2. More and more cyanide is added to globin hemochromogen in the presence of extra globin, the total globin concentration being 0.00004 N instead of 0.000005 N. The band moves to the red until when the cyanide concentration is 0.002 N, the band is 42 Å to the red of the band of globin hemochromogen. Doubling the cyanide concentration

TABLE I

| CN<br>N | Position of $\alpha$ band — Å to red of 5585 Å |                    |                     |
|---------|--|--------------------|---------------------|
|         | Globin = 0.0004 N                              | Globin = 0.00004 N | Globin = 0.000005 N |
| 0       | 0  | 0                  | 0                   |
| 0.0001  | 18   | 18                 | 16                  |
| 0.0002  | 27   | 28                 | 26                  |
| 0.0004  | 33   | 34                 | 33                  |
| 0.001   | 39   | 40                 | 44                  |
| 0.002   | 41   | 42                 | 49                  |
| 0.004   | 43   | 43                 |                     |
| 0.01    |  | 48                 |                     |
| 0.02    | 42   | 57                 |                     |

TABLE II

| CN<br>Globin | Position of $\alpha$ band — Å to red of 5585 Å |                    |                    |
|--------------|--|--------------------|--------------------|
|              | Globin = 0.00008 N                             | Globin = 0.00004 N | Globin = 0.00002 N |
| 0            | 0  | 0                  | 0                  |
| 125          | 45   | 43                 |                    |
| 250          | 48   | 48                 | 48                 |
| 500          | 57   | 57                 | 57                 |
| 1,000        | 69   | 66                 | 65                 |
| 50,000       |  |                    | 100                |
|              |  |                    | No globin          |
|              |  |                    | 100                |

(making it 0.004 N) now causes no further change in the spectrum. But the addition of still more cyanide causes the band to move to the red again. (See Curve II.)

3. Finally, more and more cyanide is added to globin hemochromogen in the presence of a concentration of globin ten times greater still, namely 0.0004 N. Until the cyanide concentration is 0.004 N the

results are *precisely* the same as with the solutions containing ten times less globin. But whereas in the more dilute globin solution further addition of cyanide causes the band to shift to the red, in the solution 0.0004 N in respect to globin the cyanide concentration must be increased from 0.004 N to 0.04 N before the band is shifted to the red again. (See Curve III.)

4. Whenever the globin concentration is *greater* than 0.00004 N, the cyanide concentration is *less* than 0.002 N, and the band is accordingly *less* than 42 Å to the red of the band of globin hemochromogen, the position of the band depends solely on the cyanide concentration. Increasing the globin concentration has no effect on the spectrum so long as the cyanide concentration is kept constant.

5. Whenever the cyanide concentration is *greater* than 0.002 N and the band is *more* than 42 Å to the red of the band of globin hemochromogen, the position of the band depends solely on the *ratio* of the concentrations of cyanide and globin. Increasing the cyanide concentration has no effect on the spectrum provided only that the globin concentration is increased to the same extent.

6. The nature of the mixed spectrum given by a solution containing two different pigments depends on how far apart are the bands of the pigments. If the bands are far enough apart they do not fuse completely but can be distinguished separately in the mixed spectrum. For instance, the  $\alpha$  bands of the first and second cyanide compounds of reduced heme which are 150 Å apart can both be seen in the spectrum given by a mixture of the two cyanide compounds (Anson and Mirsky, 1928). If the two bands of the two pigments are close enough together, however, they fuse to give a single band whose maximum absorption is at some wave length intermediate between the wave lengths at which the two individual pigments have their maximum absorptions. The more of one of the pigments is present the closer will the position of the intermediate band be to the band of that pigment. Thus the  $\alpha$  bands of oxy and carbon monoxide hemoglobins (Hartridge, 1912) and of globin and ammonia hemochromogens (Anson and Mirsky, 1925, 1928) fuse to give intermediate bands whose positions are determined by the compositions of the mixtures. Finally, the bands of two pigments may not be close enough to mix to give a single intermediate band and still not far enough apart for two points of maximum

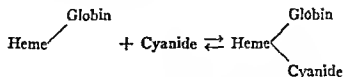
absorption to be distinguished easily in the mixed spectrum. In such cases the mixed spectrum has a band much broader than the band of a single pigment and this band becomes more and more asymmetrical as the relative concentration of one of the pigments in the mixture is increased.

In general the same mixed spectrum which is seen when one looks through a single cell containing a solution of two different pigments is also seen when one looks at the same time through two cells, one containing a solution of one pigment alone, the other containing a solution of the other pigment alone.

If one looks through two cells, one containing a solution of globin hemochromogen, the other a solution of the second cyanide compound of reduced heme, one sees a very broad asymmetrical band. It is impossible by means of optical mixtures of the spectra of globin hemochromogen and the second cyanide compound to imitate the single bands given by solutions of cyanide plus globin hemochromogen. All the spectra actually observed, however, can be imitated by means of optical mixtures of the spectra of globin hemochromogen, the second cyanide compound and the product with the band 42 Å to the red of the band of globin hemochromogen.

Two conclusions are drawn from the new facts:

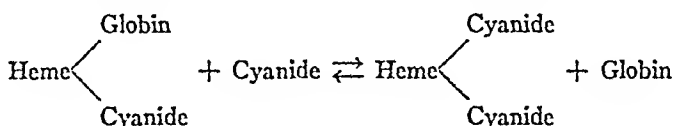
1. Cyanide can combine with globin hemochromogen without competing with or displacing globin to form a pigment with an  $\alpha$  band 42 Å to the red of the band of globin hemochromogen.<sup>1</sup> In this reaction it is not behaving as a typical nitrogenous hemochromogen-forming substance but probably like carbon monoxide.



2. Cyanide can further react with cyanide-globin hemochromogen to form the second cyanide compound of reduced heme which contains

<sup>1</sup> Hill (1929) has observed an analogous pigment obtained by adding both nicotine and cyanide to heme. He believed that every hemochromogen contains two nitrogen groups per heme and that the compound of nicotine and cyanide with heme is a typical hemochromogen which happens to contain two different nitrogen groups.

two cyanide groups per heme. In this reaction cyanide is competing with and displacing the typical nitrogenous substance, globin.



From Curve I alone one might suppose that cyanide in reacting with globin hemochromogen simply displaces the globin and forms the second cyanide compound of reduced heme. On this basis any intermediate  $\alpha$  band observed must be the result of the fusion of the  $\alpha$  bands of globin hemochromogen and the second cyanide compound. This theory is disproved by the fact that one cannot imitate the observed spectra by means of optical mixtures of the spectra of globin hemochromogen and the second cyanide compound. To explain the mere existence of single intermediate bands one must assume the existence of a new compound with a band between the bands of globin hemochromogen and the second cyanide compound. Since the compounds of heme with either globin or cyanide alone do not have such a band, the new compound probably contains both globin and cyanide.

Curve III shows that cyanide first reacts with globin hemochromogen to form a new compound with a band 42 Å to the red of the band of globin hemochromogen and only then reacts further with this new compound. The extent of formation of the new compound is independent of the globin concentration, so globin is not being displaced. The new compound therefore contains globin as well as cyanide, a conclusion already drawn from Curve I.

If the new compound contains globin, and if cyanide reacts with the new compound to form the second cyanide compound which does not contain globin, then this reaction must involve the displacement of globin by cyanide. In confirmation of this the extent to which the second cyanide compound is formed from the new compound of cyanide and globin hemochromogen depends solely on the cyanide to globin ratio, which is precisely what one would expect were the cyanide and globin competing for the heme.

The reason why Curve I deviates from Curves II and III even before the cyanide concentration reaches 0.002 N is now clear. If the globin concentration is low enough some globin is displaced by cyanide even

if the cyanide concentration is not high enough to convert globin hemochromogen completely into cyanide-globin hemochromogen. To separate the two reactions the globin concentration must be above a certain minimum. Theoretically the two reactions can never be separated completely.

The fact that cyanide can displace globin from its combination with heme is most simply explained in the assumption that cyanide can react like a typical nitrogenous substance such as globin. This assumption, however, is not a necessary one. The affinity of hemoglobin for carbon monoxide depends on the hydrogen ion concentration; carbon monoxide and hydrogen ions compete for the hemoglobin and can displace each other. Yet these two groups do not react with hemoglobin in the same way. In all probability, carbon monoxide combines with the iron and the hydrogen ion does not, but combines with a group close to the iron and so influences the affinity of the iron for carbon monoxide.

### *Experimental Procedure*

It remains to describe in detail the manner in which the values put down in the tables were obtained. In all cases the solutions were at 0°C. in a cold room, the final concentration of NaOH was 0.5 N, solid sodium hydrosulfite ( $\text{Na}_2\text{S}_2\text{O}_4$ ) was added as a reducer, and the bands read after 5 minutes with a reversion spectroscop (Hartridge, 1912). In some cases the bands were read again after several hours. No change was found. The purpose of the strong alkali was to bring out the hemochromogen-forming capacity of globin (Anson and Mirsky, 1928).

The three solutions of Table I were 0.000005 N, 0.00004 N and 0.0004 N in respect to globin. The 0.000005 N solution was prepared by adding 2.5 ml. of 0.2 N HCl to 1 ml. of 8.5 per cent horse carbon monoxide hemoglobin and then diluting with NaOH. This solution was accordingly 0.000005 N in respect to globin, heme and iron. The 0.00004 N and 0.0004 N solutions contained 0.00001 N globin and heme from hemoglobin and in addition denatured globin prepared by the oxalic acid-acetone method (Anson and Mirsky, 1930a).

The 0.00008 N solution of Table II contained 0.00002 N globin and heme from hemoglobin and in addition 0.00004 N globin prepared by the acetone method. To obtain the 0.00004 N and 0.00002 N solutions, the 0.00008 N solution was diluted with 0.5 N NaOH.

The band 42 Å to the red of the  $\alpha$  band of globin hemochromogen is a sharp band intermediate in intensity as well as in position between the  $\alpha$  bands of globin hemochromogen and the second cyanide compound of reduced heme.

When the cyanide to globin ratio reaches 1,000 the band begins to be too asymmetrical to be read with the reversion spectroscop.

Alkaline solutions of hydrosulfite attack the cements which were tried, including de Khotinsky cement. The decomposition products of the cements influence the spectra. This difficulty was avoided by using cells made of paraffin blocks and pieces of glass microscope slides. Such cells can be made in a few minutes. From paraffin blocks 1.5 cm. or 3 cm. thick depressions are melted out with a hot iron. These depressions are closed on the sides of the blocks by melting in pieces of glass. To make a double cell to permit looking through two solutions at once, a piece of glass which is to act as a partition is first melted between two blocks of paraffin. If cells only a few millimeters thick are desired, it is better to place slightly softened but not melted paraffin between two pieces of glass which are then pressed together in a vise until they are the desired distance apart. The glass sides of the cell are thus automatically made parallel. Finally some of the paraffin is melted out to form the cell.

### *The Nature of the Two Cyanide Compounds of Reduced Heme*

*A priori* there are four possibilities in regard to the nature of the two cyanide compounds of reduced heme. Neither of the two cyanide groups may be a typical nitrogenous hemochromogen-forming group; both may be; cyanide may be acting as a typical nitrogenous group in forming only the first; or in forming only the second compound. The present experiments do not decide conclusively which of these four possibilities corresponds to the truth. What has been made clear is that cyanide can combine with heme in two different ways. In one reaction it does not compete with or displace globin; in the other it does. In the light of these results and of the fact that the spectrum of the first compound is similar to that of globin hemochromogen and the spectrum of the second compound is similar to that of cyanide globin hemochromogen, the simplest theory is that the first compound is a typical hemochromogen and the second compound a compound of cyanide with cyanide hemochromogen.

### *The Composition of Hemochromogen*

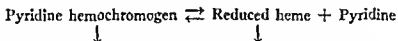
If the first cyanide compound of reduced heme which has one cyanide group per heme is a typical hemochromogen, then it is probable that hemochromogens in general contain one nitrogen group per heme. It would obviously be desirable to confirm or refute this conclusion by independent investigation of the composition of some hemochromogen whose nitrogenous substance forms only one hemochromogen-like compound with heme. The attempts to do this have not yet led to any conclusive results. Von Zeynek found by analyses of

the precipitates that ammonia hemochromogen (1898) contains one nitrogenous group and pyridine hemochromogen (1910) two nitrogenous groups per heme. But such direct analyses must remain of dubious significance until it is shown that in the preparation of the precipitates no hemochromogen-forming nitrogen groups are split off from the heme and no non-hemochromogen-forming nitrogen groups added to the carboxyl groups.

Two molecules of pyridine must be added to each molecule of heme in order to convert heme into pyridine hemochromogen (R. Hill, 1926, 1929). If all the pyridine added were combined with the heme this would mean that pyridine hemochromogen contains two pyridines per heme. But some of the pyridine added must be free to drive the equilibrium:



to the left. It cannot be decided from Hill's experiments whether or not this free pyridine is negligible. In dilute solution of the pigment the concentration of free pyridine needed to prevent visible dissociation of the hemochromogen may be determined. It is not known whether the same concentration of free pyridine is needed to prevent visible dissociation of the hemochromogen in the concentrated solutions actually used. As has already been pointed out (Mirsky and Anson, 1929) the equilibrium is complicated by the aggregation and precipitation of both pyridine hemochromogen and reduced heme:



Precipitation or aggregation of the hemochromogen would drive the equilibrium to the left and so reduce the concentration of free pyridine needed to prevent detectable dissociation of the hemochromogen. Precipitation of the heme would have the opposite result. It is not known what effect concentration has on the relative velocities and extents of the aggregation of the two pigments.

Dr. Northrop has suggested that it might be possible to estimate the free pyridine in a solution of pyridine hemochromogen by shaking the aqueous solution with a solvent which does not mix with water but in which pyridine is soluble, estimating the total pyridine in this solvent, and then calculating the free pyridine in the water from the distribution constant.



*Cyanide and Carbylamine*

Warburg, Negelein and Christian (1929) have recently described some reactions of carbylamine with hemoglobin which in some ways resemble the reactions of cyanide with heme. Carbylamine can combine with hemoglobin in two different ways. In one reaction it competes with carbon monoxide, in the other it does not. Native globin in hemoglobin is not behaving like a typical nitrogenous substance such as ammonia or denatured globin. Whether one of the carbylamines is behaving like a typical nitrogenous substance has not yet been determined.

*Conclusions*

Cyanide can react with globin hemochromogen in two different ways. In the first reaction cyanide combines with globin hemochromogen without displacing or competing with globin. In the second reaction cyanide displaces globin.

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# THE EFFECT OF LOW PRESSURES ON CELL OXIDATION

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## INTRODUCTION

It has long been a matter of common knowledge that not only human beings but most other animals as well as plants can endure great variations in their supply of oxygen without obvious ill effects. This is demonstrated by the existence of life at high altitudes, the phenomena of anaerobiosis and many other facts. In addition to the fundamental researches of Harden, Hill, Meyerhof, Warburg, and many others, which have demonstrated the difference between, and yet the interdependence of aerobic and anaerobic respiration, numerous attempts have been made to correlate the amount of oxygen present with the degree of activity of an organism. One of the most recent of these is that of Amberson (1928) who shows quite definitely that in the case of *Paramoccium* and fertilized *Arbacia* eggs the cell respiration continues at the normal rate, almost independent of the oxygen supply until the latter reaches an exceedingly low value (about 10 mm. Hg). Warburg (1926) also noticed the same phenomenon in the case of yeast. Most other investigators' results have agreed with these findings, among them those of Henze (1910), Lund (1918), Hamburger and St. Györgi (1925), Pütter (1924), Harvey (1925). The activities which were observed in this connection covered a wide range, including the actual uptake of oxygen itself (Amberson, Warburg). Mention may also be made of investigations concerning the effect of lowered oxygen tension on mammals (see Barcroft's book, 1925). There seems to be little doubt, therefore, that within wide but varying limits the organism is independent of the oxygen supply.

The above considerations apply to changes in oxygen tension where the tension is varied by taking different proportions of oxygen and nitrogen but where the total pressure of the system is held constant

at approximately 760 mm. Hg. Obviously the tension may also be varied by altering the *total pressure* where the relative gas tensions are held constant. This occurs both naturally and artificially when air is used and the barometric pressure is reduced (*e.g.*, high altitudes or low pressure chambers). The classic work of Bert (1878) shows the diversity of effects which might be obtained by a lowering (or raising) of the barometric pressure. More recently interest in this connection has been centered on the influence of these conditions on human reactions, an interest which, of course, has been stimulated by the war and the increasing importance of aviation.<sup>1</sup> Although the response of large animals to lowered pressures has been thoroughly studied very little attention has been paid to the effect of low pressures on the respiration of the individual cells and tissues themselves. The assumption has been tacitly made that the effect of a reduced oxygen tension whether secured by reducing the percentage of oxygen at atmospheric pressure, or by reducing the total pressure, would be the same. From the purely physical point of view such an attitude is very natural.

It seemed worth while, however, to test the validity of this assumption by a series of experiments in which the respiration of a simple organism would be measured under varying oxygen tension, these variations to be secured by altering *both* the percentage composition of the gases *and* the total pressure. The results of these experiments show quite clearly that there is a distinct and in some cases decided difference in the effect of gas tension depending on how the changes in tension are made. Or, in other words, it may be said that a reduced pressure, has, *per se*, an effect on tissue oxidation which is not obtained with a reduction of simply the oxygen tension.

### Methods

The organism used in this work was baker's yeast, a fresh suspension of which was made up for each experiment in Ringer solution without bicarbonate, but containing 1 per cent glucose. The gas exchange was measured by means of a modification of the Warburg manometric method. The simple manometer, as well as the differential mano-

<sup>1</sup> J. Barcroft in his book, "The Respiratory Function of the Blood" (1925) and E. C. Schneider in a series of papers in the *American Journal of Physiology* have summarized what has been accomplished in this field.

meter, have been described fully in Warburg's book (1926), together with details regarding the shaking device and temperature regulation. The only modification of the method was made when it became necessary to measure oxygen absorption or carbon dioxide evolution under reduced barometric pressure.

For this purpose the simple manometer is clearly not adapted, since it is open on one limb. It is also impossible to use the differential manometer in its usual form because both limbs, or the containers attached thereto, must be initially at the same pressure. Therefore a special differential manometer was constructed. The manometer proper was of the usual type, very similar to illustration on page 1 of Warburg's book ("Über den Stoffwechsel der Tumoren, 1926"), with posterior extensions carrying two cups or vessels. Each of these was of the "Kegelgefäß" or "conical vessel" type with a side extension and a small insert of glass on the center of floor of the vessel for carrying alkali. Each of the side extensions was fitted with a ground-in glass stopper. All joints were carefully ground and greased and their resistance to leakage carefully checked. On the upward extension of each manometer limb there was a glass stopcock, *A* and *B*. Then the two extensions were joined by fused glass tubing in the form of an inverted *Y*, at the top of which was a three-way stopcock, *C*. Since the system was all of glass the only leaks could occur at the stopcocks. These were accordingly specially ground in, greased, and tested frequently against leakage.

The fluid used in the manometer was Brodie's solution. In order to provide for the introduction of the fluid it was necessary to open the bottom of the manometer and fuse on a piece of tubing with another stopcock. When the latter was opened the fluid could be run in to the desired level from below and the stopcock closed. The manometer then functioned in the usual manner. The glass part was fastened securely to a wooden back and carried on the shaker as described by Warburg. At the beginning of an experiment the desired amount of yeast suspension was placed in one of the vessels and an equal amount of Ringer solution in the other together with any other material needed. The vessels were then placed on the manometer and held in place by springs. If a special gas mixture was to be investigated, all the upper stopcocks (*A*, *B*, and *C*) were opened and the small stoppers on the side extensions of the vessels were removed. The gas was then allowed to flow through the entire apparatus from a tank or other source of supply as described by Warburg (1926, p. 3). Stopcock *C* was then closed and the small stoppers of the vessels replaced. This isolated the system *as a whole* from the outside air. But the two vessels remained in communication with each other through the arms of the inverted *Y*. The vessels were then placed in the water bath at 37°C. and shaken till temperature equilibrium had been attained. At this point stopcock *C* was opened for an instant to relieve the positive pressure generated by the heating of the gas inside the system. Then stopcocks *A* and *B* were closed. This cut off the communication at the top between the two vessels and a differential alteration of pres-

sure could then be observed on the manometer scale as in the case of any differential manometer.

Suppose now after taking several readings it was desired to reduce the pressure within the system. The stopcocks *A* and *B* were carefully opened thus equalizing the pressure in the two vessels. A heavy rubber pressure tube was attached to the outlet above stopcock *C* and a slight negative pressure put on. Cock *C* was then opened and the pressure reduced to any predetermined amount. Then cock *C* was closed and the same method used as under atmospheric pressure. If the pressure was very low it was usually necessary to repeat this procedure two or three times because after reduction the escape of gases from solution might alter the tension of the gas space of the vessels.

To obtain reduced pressures an oil vacuum pump was attached to a 10 gallon carboy. A line ran from the latter to the heavy tubing which could be attached to the manometer. In this line was included a mercury manometer, scaled in millimeters. Several heavy glass stopcocks variously placed allowed for cutting off the line at any desired point. All joints were paraffined and shellacked. In fact the system would hold a high vacuum without appreciable change for weeks. The carboy was exhausted as highly as the pump would permit. This insured as low pressures as were ever needed. After the stopcocks in front of and behind the carboy had been closed the carboy served as a reservoir of low pressure to be drawn on when desired.

For experiments with carbon monoxide it was necessary to generate the gas. This was done by the method described in Treadwell's "Analytical Chemistry" (11th German edition, 1923, vol. 2, p. 669), in which formic acid is run slowly into hot concentrated  $\text{H}_2\text{SO}_4$ . The gas formed was run through a condenser and flasks of water and soda lime, to remove water vapor and carbon dioxide. It was then mixed in desired preparations with oxygen in a gasometer and thence run through the manometer in the usual way.

The results may be expressed either on an absolute or relative basis. If the latter, the normal respiration may be called 100 per cent and any deviation expressed as a per cent of this normal. This method is often most convenient for comparative data in which the absolute quantities involved are of secondary consequence. However, for purposes of calculation the absolute amounts must be known. In this case we may use as units the number of cubic millimeters oxygen taken up or carbon dioxide evolved per milligram fresh weight of yeast per hour. If we wished to compare different samples of yeast it would be necessary to use the dry weight, but since in this work the same yeast was used throughout any one experiment the fresh weight may be used. Then any two experiments may be compared on a percentage basis.

Warburg (1926, pp. 8-10) has developed a formula for the calculation of volume changes of gas in the differential manometer. He uses the following quantities:

$P$  = Initial pressure in both vessels.

$P_o$  = The normal pressure (*i.e.*, 760 mm. Hg) or 10,000 mm. Brodie.

- $h$  = Observed difference of level in the manometer fluid.  
 $h'$  = Increase in pressure in the compensation vessel.  
 $\Delta p$  = Increase in pressure in the experimental vessel.  
 $A$  = Cross section of the capillary.  
 $V_G$  = Gas space in the experimental vessel.  
 $V_{G'}$  = Same in compensation vessel.  
 $V_F$  = Liquid space in the experimental vessel.  
 $V_{F'}$  = Same in compensation vessel.  
 $T$  = Absolute temperature.  
 $\alpha$  = Absorption coefficient of the gas in the experimental vessel.  
 $\alpha'$  = Same in compensation vessel.  
 $x$  = cmm.  $O_2$  or  $CO_2$  absorbed or evolved.

The increase of gas in the gas space of the experimental vessel is:

$$\frac{P + \Delta p}{P_*} \frac{273}{T} \left( V_G + A \frac{h}{2} \right) - \frac{P}{P_*} \frac{273}{T} V_G \dots \dots \dots (1)$$

If the value of  $A$  is small then expression (1) becomes

$$\Delta p \left( \frac{V_G \frac{273}{T}}{P_*} + \frac{A}{2} \frac{273}{T} \frac{P}{P_*} \right) \dots \dots \dots (2)$$

He next assumes that  $P$  as a rule will equal  $P_*$  and therefore  $\frac{P}{P_*} = 1$ . ("Hier ist

$\frac{A}{2} \frac{273}{T} \frac{P}{P_*}$ , von der Grösse eines Korrektionsgliedes. Bedenken wir, dass  $P$  in der

Regel nahezu gleich  $P_*$  ist, so können wir ohne merklichen Fehler den Faktor  $\frac{P}{P_*}$

. . . . gleich 1 setzen" . . . . p. 9.) Therefore expression (2) reduces to:

$$\Delta p \left( \frac{V_G \frac{273}{T}}{P_*} + \frac{A}{2} \frac{273}{T} \right) \dots \dots \dots (3)$$

Furthermore the value of  $\Delta p$  is found to be

$$h \left( 1 + \frac{\frac{A}{2} \cdot \frac{273}{T}}{\frac{V_{G'} \frac{273}{T} V_{F'} \alpha'}{P_*}} \right) \dots \dots \dots (4)$$

from which, in the numerator of the fraction within the parenthesis, the term  $P_*$  has likewise been eliminated. In dealing with low pressures the assumption obviously does not hold that  $P$  approximates  $P_*$ . In fact it is usually widely differ-

ent. Hence the factor  $\frac{P}{P_o}$  must be retained. The entire equation then is as follows:

$$x = h \left[ \left( 1 + \frac{\frac{A}{2} \cdot \frac{273}{T} \cdot \frac{P}{P_o}}{\frac{V_{G'} \frac{273}{T} + V_{F'} \alpha'}{P_o}} \right) \left( \frac{V_G \frac{273}{T} + V_F}{P_o} + \frac{A}{2} \cdot \frac{273}{T} \cdot \frac{P}{P_o} \right) \right] \dots (5)$$

The term enclosed by brackets in expression (5) is the so-called "Gefässkonstant" or "vessel constant" which, when multiplied by the observed change in the manometer fluid level, gives the amount of gas exchange in cubic millimeters. In this term the value  $P$  occurs in the numerator twice. Therefore the magnitude of the term will vary directly as  $P$ . In other words, as the pressure in the system is lowered the vessel constant will decrease and *vice versa*. Thus if an organism absorbs the same number of molecules of oxygen per unit time as the pressure is lowered then the observed pressure change,  $h$ , must increase to compensate for the decrease in the vessel constant.

There are certain technical details which should be noted. The fluid in the manometer was Brodie's solution, used because the value of  $P_o$  expressed in millimeters Brodie is an even 10,000. The volumes of the two sides of the manometer were determined by filling each side separately with mercury and weighing. The diameter of the capillary was similarly determined. The stopcocks were greased with a petroleum jelly-rubber composition which was firm enough to prevent leakage of air. Prior to actual use, the whole apparatus was set up empty in the water bath, exhausted as completely as possible, and shaken 2 hours. During this time no change in the fluid level of the manometer could be observed.

## RESULTS

*The Respiration and Fermentation of Yeast.*—When the oxygen uptake of yeast is measured at different tensions the result is similar to that found by other investigators. There is little or no diminution in respiration until quite low tensions are reached. On the other hand, if the pressure is reduced the respiration falls off much more rapidly. This is illustrated by the data in Tables I and II.

In Tables I and II it is evident that the oxygen uptake decreases more rapidly when the pressure is reduced than when the pressure is maintained constant and the per cent of oxygen is cut down. A particularly clear instance of this occurs in Table II with a tension of 36 mm. of oxygen. In 5 per cent oxygen at normal pressure the

respiration is 84 per cent of that in air (144 mm.). When the pressure on a mixture containing 21 per cent oxygen, however, is reduced to 190 mm. giving the same partial pressure of oxygen (36 mm.) the respiration is cut down to 34 per cent of the normal. There is then clearly one effect on respiration which is brought about by a reduction of pressure, *per se*, and which is independent of the quantity of oxygen (or tensions).

TABLE I

3 cc. yeast suspension (10 mg. per cubic centimeter). Temperature 37°C. Initial gas mixture 100 per cent oxygen = 712 mm. oxygen tension.<sup>2</sup> 0.4 cc. 10 per cent KOH in inset.

## A

Barometric pressure maintained at 760 mm. and per cent of oxygen, exclusive of water vapor, varied by mixing with nitrogen.

|  |     |      |     |    |    |
|--|-----|------|-----|----|----|
| Per cent O <sub>2</sub> .....  | 100 | 43   | 21  | 9  | 5  |
| Tension O <sub>2</sub> in mm. Hg.....                                    | 712 | 306  | 144 | 64 | 36 |
| Respiration (cmm. O <sub>2</sub> absorbed per mg. yeast per hour).....   | 48  | 47.5 | 47  | 45 | 33 |
| Per cent of normal (i.e., of respiration under standard conditions)..... | 100 | 99   | 98  | 94 | 69 |

## B

Per cent of oxygen maintained at 100 per cent, exclusive of water vapor, and barometric pressure varied.

|   |     |     |     |     |     |     |
|---|-----|-----|-----|-----|-----|-----|
| Barometric pressure in mm. Hg.....        | 760 | 600 | 500 | 400 | 300 | 200 |
| Corresponding O <sub>2</sub> tension..... | 712 | 552 | 452 | 352 | 252 | 152 |
| Respiration (as above).....               | 48  | 48  | 44  | 38  | 36  | 16  |
| Per cent of normal (as above).....        | 100 | 100 | 92  | 79  | 75  | 33  |

Since respiration and fermentation (aerobic and anaerobic respiration) are closely related, experiments were performed to find out if the

<sup>2</sup> It is of course necessary in all these experiments to subtract the vapor tension of the water present. At 37°C. this amounts to 48 mm. Hg at all barometric pressures. For example with 100 percent O<sub>2</sub> the total pressure is 760 mm. but the oxygen tension will be 760 - 48 = 712. If this same gas is reduced to a barometric pressure of, say, 300 mm. Hg then the oxygen tension will be 300 - 48 = 252. The same principle applies when the gas mixture contains any other percent of oxygen.



lowering of the pressure affected the latter as well as the former. This might be expected if the reduced pressure attacks the oxidation system of the cell as a whole. If, however, it is confined to that portion of the

TABLE II

3 cc. yeast suspension (10 mg. per cubic centimeter). Temperature 37°C. Initial gas mixture 21 per cent oxygen, 79 per cent nitrogen (exclusive of water vapor). 0.4 cc. 10 per cent KOH in inset.

## A

Average of two experiments, expressed as per cent of respiration with the initial mixture as the normal. Barometric pressure maintained at 760 mm. Mixtures as in Table I, A.

|                                       |     |    |    |      |
|---------------------------------------|-----|----|----|------|
| Per cent O <sub>2</sub> .....         | 21  | 9  | 5  | 1.6  |
| Tension O <sub>2</sub> in mm. Hg..... | 144 | 64 | 36 | 11.5 |
| Per cent of normal respiration.....   | 100 | 97 | 84 | 60   |

## B

Average of five experiments, expressed in per cent of respiration in this mixture at 760 mm. Per cent of gases in the mixture, exclusive of water vapor: oxygen 21 per cent, nitrogen 79 per cent. Barometric pressure varied.

|   |     |     |     |     |     |
|---|-----|-----|-----|-----|-----|
| Barometric pressure in mm. Hg.....        | 760 | 380 | 190 | 115 | 80  |
| Corresponding O <sub>2</sub> tension..... | 144 | 70  | 30  | 13  | 6.5 |
| Per cent normal respiration.....          | 100 | 74  | 34  | 27* | 7** |

\* One experiment.

\*\* Average of three experiments.

TABLE III

3 cc. yeast suspension (10 mg. per cubic centimeter). Temperature 37°C. Initial gas nitrogen. Barometric pressure varied.

|   |      |     |     |     |      |
|---|------|-----|-----|-----|------|
| Barometric pressure in mm. Hg.....  | 760  | 540 | 415 | 250 | 135  |
| Respiration: cmm. CO <sub>2</sub> produced per mg. per hour.....                  | 45.5 | 46  | 45  | 45  | 45.5 |
| Per cent of normal (i.e., per cent of respiration under standard conditions.....) | 100  | 101 | 99  | 99  | 102  |

system which involves simply the uptake of oxygen then there might or might not be an effect on the anaerobic oxidations. Table III is an example of the results obtained. In this type of experiment the

manometer was filled with nitrogen gas which had been run through alkaline pyrogallol to remove all but the last traces of any oxygen that might be present. Such traces as remained would not affect the result materially. No KOH was placed in the vessel and the production of  $\text{CO}_2$  was measured.

TABLE IV

3 cc. of 2 per cent fructose (Pfanstiel) dissolved in  $\text{N}/2 \text{ Na}_2\text{CO}_3$ . Temperature  $37^\circ\text{C}$ .

## A

Initial gas 100 per cent  $\text{O}_2$ . Pressure maintained at 700 mm. Per cent of oxygen varied.

|   |     |     |     |    |      |
|---|-----|-----|-----|----|------|
| Per cent of oxygen.....   | 100 | 21  | 9   | 5  | 1.6  |
| Oxygen tension in mm. Hg.....   | 712 | 144 | 64  | 36 | 11.5 |
| Cmm. $\text{O}_2$ per hour absorbed by the fructose.....                                | 530 | 329 | 180 | 81 | 12.5 |
| Per cent absorption based on 100 per cent $\text{O}_2$ and 760 mm. as 100 per cent..... | 100 | 62  | 33  | 15 | 2.5  |

## B

Initial gas 100 per cent  $\text{O}_2$ , exclusive of water vapor, maintained. Pressure varied.

|   |     |     |     |    |
|---|-----|-----|-----|----|
| Barometric pressure.....  | 760 | 345 | 195 | 80 |
| Oxygen tension in mm. Hg.....   | 712 | 297 | 147 | 32 |
| Cmm. $\text{O}_2$ per hour absorbed by fructose.....                                    | 492 | 369 | 324 | 92 |
| Per cent absorption based on 100 per cent $\text{O}_2$ and 760 mm. as 100 per cent..... | 100 | 73  | 66  | 19 |

## C

Both per cent of oxygen in nitrogen, exclusive of water vapor, and the barometric pressure were simultaneously varied so as to bring the oxygen tension in all cases to 63 to 64 mm. Hg. The variation in the tension was not over 1 per cent.

|                                     |      |     |     |      |
|-------------------------------------|------|-----|-----|------|
| Per cent oxygen.....                | 9    | 20  | 43  | 100  |
| Barometric pressure in mm. Hg. .... | 760  | 360 | 190 | 110  |
| Oxygen tension in mm. Hg....        | 63.5 | 63  | 63  | 63.5 |
| Cmm. oxygen absorbed per hour.....  | 213  | 175 | 170 | 188  |

This experiment, which was checked several times, shows that there is no such diminution of anaerobic respiration with reduced pressure as is experienced with aerobic respiration. In fact there may be said to be no effect at all. The significance of this result is discussed below.

*Artificial Systems.*—The question arises: Is the different action of

reduced oxygen *tension* and reduced *pressure* due to a purely physical cause having to do with the solution and diffusion of the gas, or is it dependent upon the organization of the cell itself? In this connection the usual type of experiment was performed with two artificial oxidation systems which proceed in homogeneous solution: the absorption of oxygen by fructose in alkaline solutions and the oxidation of cysteine. These systems have been studied exhaustively by Warburg and his co-workers who have shown that both are oxidations catalyzed by metals.<sup>3</sup> (Sakuma, 1923; Warburg and Yabusoe, 1924; Krebs, 1927).

The results are indicated in the data cited in Tables IV and V.

The results shown in Table IV indicate that, apart from experimental error, the amount of oxygen uptake with fructose is dependent on the oxygen tension alone and is not affected materially by

TABLE V

25 mg. cysteine (Pfanstiehl) in 3 cc. borate-KOH buffer at pH 9.3. Temperature 20°C. Initial gas 100 per cent oxygen, exclusive of water vapor.

|   |      |      |      |
|---|------|------|------|
| Per cent oxygen.....                                | 100  | 100  | 43   |
| Barometric pressure in mm. Hg.....                  | 760  | 363  | 760  |
| Oxygen tension in mm. Hg.....                       | 712  | 315  | 306  |
| Cmm. oxygen absorbed per hour per mg. cysteine..... | 25.4 | 12.6 | 12.7 |

changes of pressure. In other words the initial *concentration* of oxygen is the determining factor.

The typical experiment cited in Table V indicates that the cysteine system behaves analogously to the fructose system in that the oxidation is dependent solely on the oxygen tension.

*The Inhibition of Respiration by Carbon Monoxide.*—The long series of well known facts concerning the action of carbon monoxide on hemoglobin and its capacity to inhibit tissue oxidation (Warburg, 1926) lends support to the idea that we have, in carbon monoxide, a substance which behaves in a manner very similar to oxygen. It

<sup>3</sup> No metals were added to these systems because, although fructose and cysteine of the highest obtainable commercial purity (Pfanstiehl) were used and all possible precautions observed, there were sufficient impurities in the form of metals (chiefly iron) to catalyze the oxidations.

may be said to compete with oxygen in the series of reactions which we call oxidation. It seemed therefore advisable to see if low pressure would prevent the uptake of carbon monoxide as well as that of oxygen. This could, of course, be measured by the *inhibition of the oxygen uptake*. For just so much as the carbon monoxide replaces the oxygen in the oxidation system, by just so much is the normal absorption of oxygen diminished.

The work of Warburg (1926) indicated that when the proportion of carbon monoxide to oxygen is increased the toxicity of the former increases. Since in this investigation it was intended to reduce the pressure on a constant mixture of carbon monoxide and oxygen and thereby decrease the total quantity of both gases, it was of importance to know definitely, apart from the question of pressure, whether the effect of carbon monoxide on oxidation could be varied by keeping the ratio  $\frac{\text{CO}}{\text{O}_2}$  constant and varying the total amounts, or whether, as

is implied by Warburg's work, it is solely a matter of the ratio  $\frac{\text{CO}}{\text{O}_2}$ .

To be certain of the situation two series of experiments were performed: first a series where the ratio of carbon monoxide to oxygen was kept constant but the total quantity of both was altered at 760 mm., and secondly a series where the ratio was altered. In both these series the respiration at a given oxygen tension with carbon monoxide had to be compared with the respiration at the same oxygen tension where nitrogen replaced the carbon monoxide. This, of course, was to control the normal moderate reduction of respiration due to the diminished oxygen tension itself.

In the first series (Table VI) an initial mixture of 21 per cent  $\text{O}_2$  and 79 per cent  $\text{N}_2$  was used to determine the normal respiration. Then a mixture of 21 per cent  $\text{O}_2$ , 79 per cent  $\text{CO}$  was substituted. The latter was then diluted with nitrogen to reduce the total concentration of both oxygen and carbon monoxide to the same degree.

In the second series (Table VII) the ratio  $\frac{\text{CO}}{\text{O}_2}$  was changed by filling the manometer successively with different mixtures of the two gases.

Here it will be seen that replacing 79 per cent nitrogen with an equivalent amount of carbon monoxide reduced the oxygen uptake

TABLE VI

3 cc. yeast, 10 mg. per cubic centimeter 0.4 cc. 10 per cent KOH in inset. Temperature 37°C. Initial mixture 21 per cent O<sub>2</sub>; 79 per cent N<sub>2</sub>, exclusive of water vapor. Pressure maintained at 760 mm. Average of two experiments.

| Gas mixture.....  | 21% O <sub>2</sub><br>79% N <sub>2</sub> | 21% O <sub>2</sub><br>79% CO | 10% O <sub>2</sub><br>40% CO<br>50% N <sub>2</sub> | 5% O <sub>2</sub><br>20% CO<br>75% N <sub>2</sub> |
|---|--|------------------------------|--|---|
| Oxygen tension in mm. Hg.....   | 144                                      | 144                          | 72   | 36  |
| Per cent respiration on basis of 21 per cent O <sub>2</sub> in N <sub>2</sub> , as 100 per cent.... | 100                                      | 54                           | 50   | 36  |

TABLE VII

Comparison of mixtures of oxygen with nitrogen and carbon monoxide. Temperature 37°C. Pressure maintained at 760 mm. Hg. 3 cc. yeast, 10 mg. per cubic centimeter. 0.4 cc. 10 per cent KOH in inset.

| Gas mixture.....  | 43% O <sub>2</sub><br>57% N <sub>2</sub> | 21% O <sub>2</sub><br>79% N <sub>2</sub> | 9% O <sub>2</sub><br>91% N <sub>2</sub> | 5% O <sub>2</sub><br>95% N <sub>2</sub> |
|---|--|--|---|---|
| Oxygen tension in mm. Hg.....   | 306                                      | 144                                      | 64                                      | 36                                      |
| Respiration in cmm. oxygen absorbed per hour per mg. yeast....  | 39.7                                     | 39.0                                     | 37.3                                    | 27.5                                    |
| Per cent respiration on basis of 43 per cent O <sub>2</sub> 57 per cent N <sub>2</sub> as 100 per cent..... | 100                                      | 98                                       | 94                                      | 69                                      |
| Gas mixture.....  | 43% O <sub>2</sub><br>57% CO             | 21% O <sub>2</sub><br>79% CO             | 9% O <sub>2</sub><br>91% CO             | 5% O <sub>2</sub><br>95% CO             |
| Ratio $\frac{\text{CO}}{\text{O}_2}$ .....  | 1.33                                     | 3.77                                     | 10.1                                    | 19.0                                    |
| Oxygen tension in mm. Hg.....   | 306                                      | 144                                      | 64                                      | 36                                      |
| Respiration in cmm. oxygen absorbed per hour per mg. yeast....  | 35.9                                     | 27.5                                     | 14.8                                    | 9.0                                     |
| Per cent respiration on basis of 43 per cent O <sub>2</sub> 57 per cent N <sub>2</sub> as 100 per cent..... | 90.5                                     | 69.5                                     | 37.4                                    | 22.7                                    |
| Per cent inhibition* .....  | 10.5                                     | 29.5                                     | 60.5                                    | 67                                      |

\* Per cent inhibition is represented by the expression:

$$\frac{100 (\text{per cent respiration in N}_2 - \text{per cent respiration in CO})}{\text{per cent respiration in N}_2}$$

to about 54 per cent. Further reduction of the concentration of both gases in equal degree at constant pressure causes an added reduction. But this may be accounted for on the basis of reduction of *oxygen*

*tension alone.* If we call the oxidation in the presence of 21 per cent  $O_2$ , 79 per cent CO equal to 100 per cent then with 10 per cent  $O_2$ , 40 per cent CO it is 92.5 per cent and with 5 per cent  $O_2$ , 20 per cent CO it is 67 per cent. This is slightly greater than, but of the same order of magnitude as, what would be expected on the basis of the oxygen-nitrogen mixtures previously investigated (Table I). Therefore it may be said that if the ratio remains constant the inhibition remains sensibly the same.

What happens when the ratio is altered may be seen in Table VII where it is very evident that the inhibitory effect of carbon monoxide

TABLE VIII

3 cc. yeast (10 mg. per cubic centimeter). Initial mixtures 21 per cent  $O_2$ , 79 per cent  $N_2$  and 21 per cent  $O_2$ , 79 per cent CO (exclusive water vapor). Pressure varied. Temperature  $37^\circ C$ . Average of four experiments.

|   |                        |      |      |      |
|---|------------------------|------|------|------|
| Gas mixture.....  | 21% $O_2$<br>79% $N_2$ | same | same | same |
| Barometric pressure in mm. Hg.....  | 760                    | 380  | 190  | 80   |
| Oxygen tension in mm. Hg.....   | 144                    | 70   | 30   | 6.5  |
| Per cent respiration on basis of 21 per cent $O_2$ , 79 per cent $N_2$ as 100 per cent. | 100                    | 71   | 31   | 7.5  |
| Gas mixture.....  | 21% $O_2$<br>79% CO    | same | same | same |
| Barometric pressure in mm. Hg.....  | 760                    | 380  | 190  | 80   |
| Oxygen tension in mm. Hg.....   | 144                    | 70   | 30   | 6.5  |
| Per cent respiration on basis of 21 per cent $O_2$ , 79 per cent $N_2$ as 100 per cent. | 56                     | 46   | 27.5 | 8    |
| Per cent inhibition .....   | 44                     | 36   | 14   | 0    |

increases as the ratio  $\frac{CO}{O_2}$  increases and *vice versa*. When the pressure is reduced on a constant mixture of two such gases of low solubility their ratio in solution will remain practically constant. If the reduced pressure has an equal effect on the reactions of the cell itself with respect to the two gases the degree of inhibition on the part of the carbon monoxide will not be altered. If on the other hand, the degree of inhibition is altered it may be taken as an indication that low pressures affect the reactions of the two gases differentially in the cell.

We may now proceed to what happens when the pressure is reduced.

The data covering this situation are summarized in Table VIII. In all experiments two parallel series were run, first with 21 per cent  $O_2$ , 79 per cent  $N_2$ , then, on the same yeast with 21 per cent  $O_2$ , 79 per cent CO. The inhibition due to the carbon monoxide was then calculated.

From Table VIII one significant conclusion may be drawn. When the pressure is reduced the inhibition progressively disappears until it may be said to be entirely removed. In others words, low pressures have a greater effect on carbon monoxide than on oxygen with respect to their relative power to affect the oxidation system of the cell.

#### DISCUSSION

There are certain theoretical considerations which may be discussed with regard to the foregoing experimental evidence without attempting to build up any complete hypothesis to account for the effect of low pressures on cell oxidations.

1. That living oxidation systems are affected by low pressures to the exclusion of artificial systems has been shown by the fact that the respiration of yeast is reduced by low pressures over and above what can be accounted for by simple decrease in oxygen concentration (tension) whereas such low pressures seem to have no *extra* effect whatever on artificial systems of the fructose or cysteine type. The rate of the latter is governed solely by the number of oxygen molecules present. This proves nothing but what is already universally admitted, that in the complex and heterogeneous oxidation system of the cell there is a mechanism which cannot be duplicated in all respects by a relatively simple, homogeneous, system *in vitro*. We may however, narrow down the field to some extent, for any purely concentration effects due to the action of reduced pressure on a homogeneous system may be ruled out.

2. The lack of any influence of low pressures on the anaerobic respiration of the yeast indicates that not all of the oxidation system of the cell is affected, but only that part which has to do with the uptake of oxygen. We must assume the existence of some kind of catalyst in the cell which facilitates this uptake either by direct union with oxygen, or by causing it to combine, temporarily at least, with some substance from which the oxygen can be carried to the final

oxidisable materials in the cell. The possibility of an organic peroxide immediately suggests itself, or a haem derivative on the order of hemoglobin or Warburg's "Atmungsferment." It is not necessary to postulate any definite chemical entity but is sufficient to assume merely the presence of some substance with which the oxygen combines.

It is known that some peroxides such as hydrogen peroxide are relatively stable in contact with inert gases like nitrogen at normal pressure, but are very easily decomposed if the pressure is lowered. The equilibrium between hemoglobin and the oxygen tension has likewise been elucidated although in this instance it is generally understood that the equilibrium is the same regardless how the partial pressure of the oxygen is achieved (whether by gas mixtures or by reduction of the barometric pressure). At all events the suggestion may be made that one of the intermediate compounds of oxygen in the cell is of such a nature that it is broken up to a greater or less extent by lowering the barometric pressure. Conversely, of course, a low pressure would prevent the formation of some of this compound in the first place and would thus decrease the amount of oxygen which could eventually be used by the cell.

3. The data secured with carbon monoxide are of interest in this connection. Since it has been shown quite definitely that the degree of inhibition on the part of carbon monoxide depends upon the ratio  $\frac{\text{CO}}{\text{O}_2}$  the differential effect of low pressure must mean that, in the cell itself, at the point where oxidation is taking place, the ratio must be altered by low pressure. If we make the further assumption that the intermediate compound previously mentioned may be formed with carbon monoxide as well as oxygen then we have only to say that low pressures decompose the compound with carbon monoxide more readily than that with oxygen. Warburg and Negelein (1928) have proved quite conclusively that there exists a CO- "Atmungsferment" compound which is decomposed by light. This is itself evidence of a relatively unstable compound. It seems also reasonable to suppose that this, or a similar, substance may be decomposed by low pressure.



## SUMMARY

1. A method is described for measuring tissue oxidation under reduced barometric pressure.

2. The oxygen uptake of yeast is diminished by low barometric pressures to a greater extent than by a reduction of the partial pressure of oxygen, to a corresponding degree, at atmospheric pressure.

3. This effect of low pressure is not observed with certain *in vitro* oxidation systems.

4. The anaerobic respiration (carbon dioxide production) of yeast is not at all affected by low pressures.

5. The inhibition of tissue oxidation caused by carbon monoxide is removed by lowering the pressure.

I wish to take this opportunity to thank the Board of Research of the University of California for its generous financial assistance in connection with this investigation.

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# CHEMICAL STIMULATION BY ALCOHOLS IN THE BARNACLE, THE FROG AND PLANARIA

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The several types of stimulation<sup>1</sup> are usually classified according to the nature of the environmental agent acting upon a receptor, and the classificatory terms "photoreception," "mechanical excitation," or "chemical stimulation" are used merely to indicate that the stimulation has been initiated by a certain type of stimulating agent. Subsequent events within the receptor may or may not be similar to the first effect at the receptive surface. All of the processes at the receptive surface and within the receptor involve the redistribution or the transformation of energy. It should therefore be possible to analyse the successive steps in stimulation and reception by tracing the energy changes. Studies on photic stimulation (Hecht, 1919-20, 1920-21, 1922-23, 1926-27, 1927-28, 1928-29, and 1929, and papers quoted therein), and on mechanical stimulation (Crozier, 1923-24; Crozier and Pincus, 1926-27, 1927-28, 1929-30; Crozier and Stier, 1927-28, 1928-29 and papers quoted therein; Adrian and Zotterman, 1926, and

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<sup>1</sup> "Stimulation" as used here refers to the first effects of an environmental agent upon a receptive surface, which initiate a catenary series of processes within the receptor. The final result of such receptor processes is the release of energy in some form to an adjacent material, membrane, or other cellular or non-cellular structure, which in turn transmits effects bringing about the eventual "response" or "reaction." In animals with a nervous system, the receptor processes eventually release nervous impulses.

papers quoted therein), have furnished generalized principles upon which interpretative analyses of these types of stimulation may be based. For chemical stimulation it has been difficult to formulate such principles satisfactorily, because of the incompleteness of suitable data, and because of the confusing and contradictory nature of the data already reported.

In our attempt to secure data on chemical stimulation we have studied the effects of the simplest alcohols (the normal primary aliphatic alcohols) on the barnacle, the frog, and *Planaria*, under experimental conditions which do not involve secondary processes, such as simultaneous stimulation by other agents, adaptation, narcosis, and the like. If the results are interpretable and lead to a logical hypothesis, it should be possible to test the hypothesis by investigating other homologous series of organic compounds. Such a procedure may lead to a generalized statement concerning the nature of the processes involved in chemical stimulation.

### Methods

For determining the stimulating efficiencies of the first four normal primary aliphatic alcohols on *Balanus tintinabulum*,<sup>2</sup> groups of young specimens from 2 to 10 mm. in basal diameter, and from 6 to 9 mm. in height, seated upon a stone or shell, were placed in a dish of running sea water as described in a previous paper on the effect of temperature in *B. balanoides* (Cole, 1928-29). The animals were properly protected from all other kinds of environmental stimuli. The sea water and the alcoholic solutions (made with sea water in 10 liter amounts) were run through a tin coil immersed in a water bath the temperature of which was held at 18.0°C.  $\pm 0.5^\circ$ . After observing that the pedal rhythm was normal in character and rate, the flow of sea water was stopped and the experimental solution was turned on at the same rate of flow (250 cc. per minute). The criterion for stimulating effect was any change in the rhythmic movements of the cirri. The nature of the change was noted and recorded, as well as the time within which it occurred. Sea water then replaced the solution and was allowed to flow at least 20 minutes before another test was made. (Full recovery from effects of the alcohols used was apparent by the end of 10 minutes.) By this method the threshold stimulating concentration of each alcohol was determined. It may be assumed that the alcohol furnished the minimum amount of energy necessary to "activate" or stimulate the

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<sup>2</sup> The experiments on *Balanus* were done at the Hopkins Marine Station, Pacific Grove, Calif., to the Director of which the senior author is indebted for many courtesies.

receptor. In all cases the time within which any irregularity in the cirral rhythm appeared was practically constant (from 10 to 20 minutes). The order of alcohols used was as follows: ethanol, propanol, methanol, butanol. The tests were repeated several times over a period of 3 weeks on three different groups of animals (250 individuals in all).

Studies on the stimulating efficiencies of the first five normal primary aliphatic alcohols were made in an analogous manner on the frog *Rana pipiens*. The frog was placed in a glass dish shielded from mechanical and photic stimuli. Tap water or the experimental solution flowed through the dish at a rate of 250 cc.  $\pm$  15 cc. per minute, and at a temperature of  $18.0^{\circ}\text{C.} \pm 0.3^{\circ}$ . The outflow was so adjusted that the liquid in the dish was 5 mm. deep. After 1 hour's acclimatization to the experimental conditions, during which "spontaneous" movements<sup>3</sup> were rare, the flow of tap water was stopped and the alcoholic solution (made up in distilled water in 3 liter amounts) started. As soon as a movement of the frog occurred the solution was replaced by tap water and the frog's skin was thoroughly washed by allowing excess water to flow through the dish. The reaction time was measured by a stop watch to 0.1 second. An interval of at least 30 minutes with tap water flowing at 250 cc. per minute was then allowed before the next test was made. Periods of much less than 30 minutes were too short to prevent adaptation of the frog to the alcohol. For example, if only 15 minutes were allowed between trials, the reaction time of the frog steadily increased until finally no response at all occurred. Since all areas of the skin exposed to the solutions were not equally sensitive, and since distribution of the solution throughout the dish was altered by changes in position of the frog, it was necessary to limit the orientation of the frog's body in respect to the current. The postero-anterior axis of the body coincided with the direction of flow. Slight change from this position resulted in increased variation in the reaction times obtained. Preliminary trials showed that all disturbing effects of simultaneous stimuli and of adaptation were reduced to a minimum by the procedure described. The response of the frog consisted of a vigorous movement of the hind legs and could rarely be confused with the so-called spontaneous movements. Among different frogs different thresholds of stimulation were usually found, but since the ratios of reaction times from any two frogs were constant it was possible to use the data obtained from different individuals by merely reducing the reactions times to a common threshold. After preliminary trials to determine a concentration of ethanol which was near the threshold value and which gave a reaction time of about 20 seconds, a series of solutions of the five alcohols<sup>4</sup> was made up beginning with methanol at 1.8 molar

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<sup>3</sup> "Spontaneous" movements are defined as any movements due to uncontrollable external or internal stimuli.

<sup>4</sup> Only alcohols of the highest purity from Eastman Kodak Company were used. Specific gravities were determined and were the best values reported in the literature.

and decreasing in molarity by one-third for each successive alcohol. Tests were then made with the five alcohols in different orders, using several different frogs over a period of 6 months. A series was run with decerebrate frogs to detect any difference in behavior. No significant variation in the results was noticeable, so that normal frogs were later used exclusively. A final series of tests were made on the frog with methanol to discover the effects of varying the concentration on the reaction time.<sup>5</sup>

Using the same method as for the frog, the effects of the alcohols on *Planaria dorotocephala* were also studied.<sup>6</sup> A smaller experimental dish was used, through which the water and the alcoholic solutions flowed at the rate of  $20 \pm 5$  cc. per minute. The temperature was  $18.0 \pm 0.3^\circ\text{C}$ . After demonstrating that *Planaria* exhibited a definite response whenever an alcoholic solution of a high enough concentration passed over it, tests were made with each of the five alcohols, at five different concentrations. The criterion of response was the beginning of locomotion. If the *Planaria* were allowed several hours of acclimatization, they remained quiescent thereafter unless stimulated. Soon after the alcohol came in contact with them, locomotion began, the reaction time depending upon the concentration of the alcohol. A complete series of tests involving at least 4 observations at each of five concentrations of each alcohol on single animals yielded uniform results. Marked variations among different individuals made it difficult to interpret the results obtained from a population of *Planaria*.

#### DISCUSSION OF RESULTS

The data from the barnacle presented in Table I and plotted in Fig. 1 indicate that the stimulating efficiencies of the successive alcohols increase about 3-fold as each  $\text{CH}_2$  group is added to the molecule, beginning with methanol. This result is analogous to the findings of Traube and others, who have studied the narcotic efficiency of alcohols and other organic compounds. (For review of the literature consult: Traube, 1927.) According to Traube's theory the concentrations of members of a homologous series of organic compounds, such as the normal primary aliphatic alcohols, necessary to produce a given effect will vary as the following geometrical series,  $1: 3^{-1}: 3^{-2}: 3^{-3}: 3^{-4} \dots$ . Such an exponential relationship may be expressed by the following equation—

$$C_1 = C_2 a^n \quad (1)$$

<sup>5</sup> For these experiments we are indebted to Mr. Leslie A. Stauber.

<sup>6</sup> All of the tests on *Planaria* were made by Mr. Alfred Margolis, to whom grateful acknowledgment is made.

where  $C_1$  is the concentration of a selected member of the series necessary to give a definite reaction,  $C_2$  is the concentration of a higher member of the series necessary to produce the same effect,  $a$  is a constant

TABLE I

Minimum stimulating concentrations of alcohols for *Balanus*. Temp. =  $18.0 \pm 0.5^\circ\text{C}$ .

| Alcohol       | Threshold concentration | Concentration calculated from equation (1) in text |
|---------------|-------------------------|--|
| Methanol..... | 0.06M                   | (0.06)M  |
| Ethanol.....  | 0.017                   | 0.02   |
| Propanol..... | 0.0067                  | 0.0066   |
| Butanol.....  | 0.0027                  | 0.0022   |

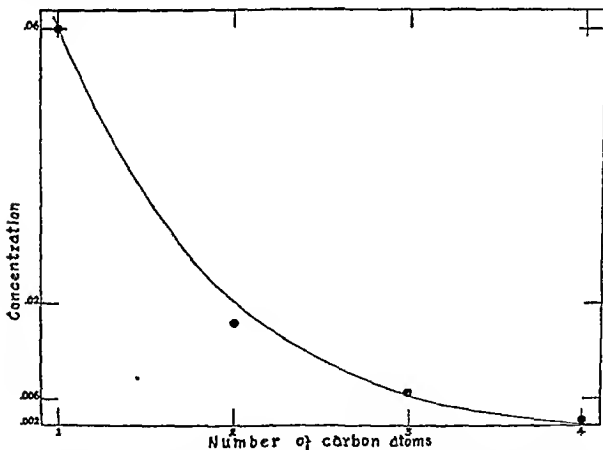


FIG. 1. Threshold stimulating concentrations of methanol, ethanol, propanol and butanol for the harnacle plotted against the number of carbon atoms in the respective molecules. The curve is exponential with the base equal to 3. The circles represent the experimental concentrations, showing very close agreement with the theoretical values.

expressing the ratio of successive concentrations, and  $n$  is the difference in the number of carbon atoms in the two molecules. The minimum stimulating concentrations of the alcohols for the barnacle calculated from equation (1), when  $a$  is 3, are very close to the threshold concentrations determined experimentally.

Proceeding on the assumption that a similar ratio would also hold for the stimulating efficiencies of the alcohols on the frog, concentrations were calculated from equation (1) and the effects of the solutions were tested.<sup>7</sup> That the receptive process measured was reversible was indicated by the relative constancy of the reaction times obtained

TABLE II

Reaction times of frog to alcohols at concentrations decreasing by one-third. Temp. =  $18.0 \pm 0.3^\circ\text{C}$ .

| Alcohol and concentration—molar |       | Number of reactions | Extreme variations | Mean  | Mean error of mean* | Mean error of mean as percentage of mean |
|---------------------------------|-------|---------------------|--------------------|-------|---------------------|--|
| Methanol                        | 1.8   | 25                  | 11.2–30.0          | 18.82 | 1.04                | 5.54                                     |
| Ethanol                         | 0.6   | 25                  | 13.2–28.3          | 20.90 | 0.98                | 4.70                                     |
| Propanol                        | 0.2   | 25                  | 11.9–33.2          | 21.30 | 1.37                | 6.44                                     |
| Butanol                         | 0.066 | 25                  | 11.5–32.0          | 24.51 | 0.94                | 3.87                                     |
| Pentanol                        | 0.022 | 25                  | 15.5–33.4          | 24.86 | 1.11                | 4.46                                     |

$$* \pm \sqrt{\frac{\sum d^2}{n(n-1)}}$$

from the tests made with each alcohol (Table II). The data show a slight but definite increase in reaction time as the length of the carbon chain increases. This increase in reaction time means that an incorrect ratio was chosen to calculate the concentrations of the various alcohols which would have the same stimulating efficiencies. In order to determine the correct ratio a knowledge of the relationship between reaction time and concentration for each alcohol is necessary, because equation (1) will hold for a given homologous series over a wide range of concentrations only if that relationship is relatively constant throughout the series. In Table III is presented the data

<sup>7</sup> A preliminary report of incomplete experiments appeared recently (Cole and Allison, 1929–30).

obtained on the frog by varying the concentration of methanol. The graphical representation of this data in Fig. 2 indicates a linear relationship between log concentration and the reciprocal of the reaction time over the range of concentrations studied, according to the following equation:

$$B = t \log \frac{C}{A} \dots \dots \dots (2)$$

where  $C$  is the concentration necessary to produce a definite reaction in time  $t$ ,  $A$  is the antilogarithm of the Y-intercept, and  $B$  is the slope

TABLE III

Reaction times of frog to different concentrations of methanol. Temp. =  $18.0 \pm 0.3^\circ\text{C}$ .

Number of reactions in each case was 15.

| Concentration—molarity | Mean reaction time—seconds | Mean error of mean as percentage of mean* |
|------------------------|----------------------------|---|
| 1.6                    | 38.9                       | 13.31                                     |
| 1.8                    | 29.8                       | 9.76                                      |
| 2.0                    | 23.3                       | 11.85                                     |
| 2.2                    | 18.8                       | 6.49                                      |
| 2.4                    | 20.3                       | 9.06                                      |
| 2.6                    | 17.4                       | 13.55                                     |
| 2.8                    | 11.9                       | 6.12                                      |
| 3.0                    | 11.4                       | 5.15                                      |

$$* \frac{100}{\text{Mean}} \times \sqrt{\frac{\sum d^2}{n(n-1)}}$$

of the line. A series of similar concentration studies with each of the five alcohols on *Planaria* exhibited the same relationship except at the highest concentration of each alcohol (Table IV). In order to make the plots more nearly coincident, as in Fig. 3 the concentrations were multiplied by the ordinate factor given in Column 5 of Table IV. At the highest concentration, the rates of reaction are slower than would be predicted from the relationship revealed by the four lower concentrations. That these deviations are real and significant is shown by the small percentage errors. Although an interpretation of this effect



is impossible without further study, it may be suggested that secondary processes at the receptor surface are responsible for the slower rates.

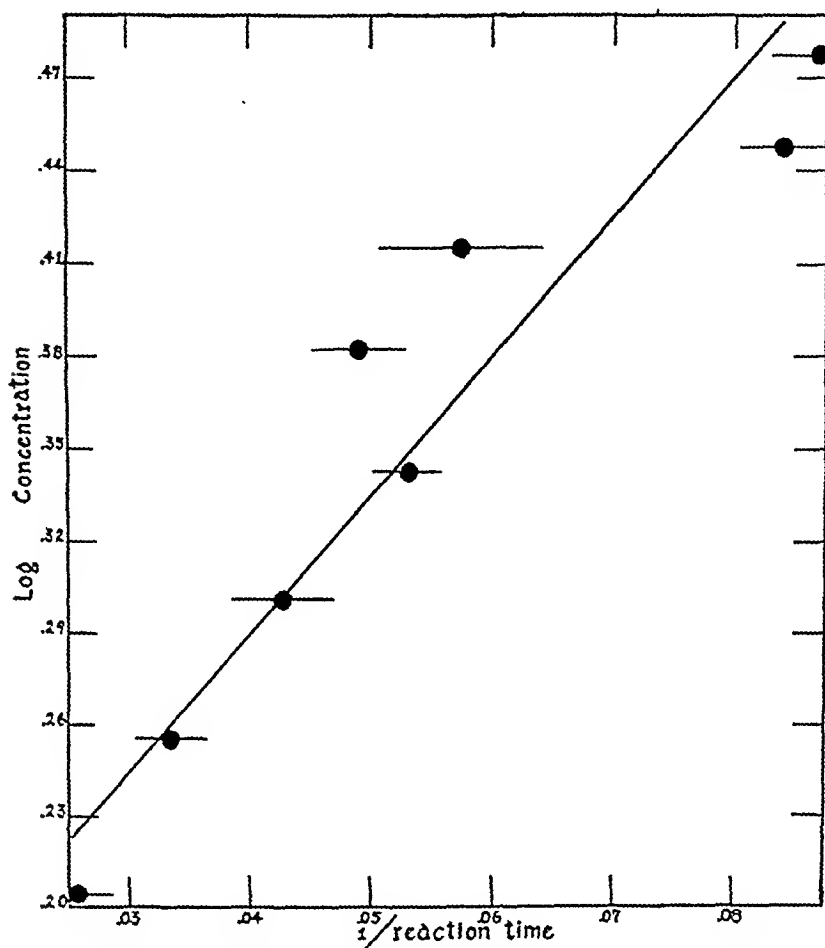


FIG. 2. Plot of reciprocal of reaction time of frog to methanol against logarithm of concentration. The length of the horizontal lines through the circles represents the magnitude of the mean error of the mean expressed as percentage of the mean. The equation of the line is  $B = t \log \frac{C}{A}$ , where the slope,  $B$ , = 4.5 and  $A$ , the anti-log of the Y-intercept, = 1.285.

If equation (2) is assumed valid for all the alcohols then the effect produced and measured is constant for all the alcohols, and is propor-

tional to  $B$ . The value of  $B$  is therefore characteristic of the receptive process initiated by members of the alcohol series in any one animal,

TABLE IV

Reaction times of *Planaria* to different concentrations of five normal primary aliphatic alcohols. Temp. =  $18.0 \pm 0.3^\circ\text{C}$ .

| Alcohol and concentration<br>—molarity |        | Number of<br>reactions | Mean reaction<br>time—seconds | Mean error of<br>mean as percent-<br>age of mean <sup>a</sup> | Ordinate factor      |
|--|--------|------------------------|-------------------------------|---|----------------------|
| Methanol                               | 0.55   | 6                      | 26.3                          | 7.31  | 1.000                |
|  | 0.60   | 11                     | 19.1                          | 6.33  |                      |
|  | 0.65   | 13                     | 17.0                          | 6.77  |                      |
|  | 0.75   | 8                      | 12.2                          | 5.16  |                      |
|  | 0.85   | 9                      | 11.5                          | 2.96  |                      |
| Ethanol                                | 0.183  | 6                      | 28.8                          | 9.20  | 2.949                |
|  | 0.200  | 9                      | 19.7                          | 3.91  |                      |
|  | 0.216  | 5                      | 16.0                          | 6.63  |                      |
|  | 0.250  | 6                      | 11.9                          | 5.63  |                      |
|  | 0.283  | 6                      | 11.7                          | 3.16  |                      |
| Propanol                               | 0.0611 | 4                      | 25.5                          | 9.06  | (3.045) <sup>b</sup> |
|  | 0.0666 | 6                      | 19.2                          | 5.94  |                      |
|  | 0.0722 | 4                      | 13.7                          | 7.01  |                      |
|  | 0.0833 | 6                      | 11.5                          | 2.78  |                      |
|  | 0.0944 | 5                      | 11.1                          | 1.98  |                      |
| Butanol                                | 0.0203 | 5                      | 29.3                          | 10.04   | (2.999) <sup>b</sup> |
|  | 0.0222 | 9                      | 18.8                          | 4.95  |                      |
|  | 0.0240 | 4                      | 17.2                          | 5.64  |                      |
|  | 0.0277 | 6                      | 12.1                          | 3.63  |                      |
|  | 0.0315 | 7                      | 9.8                           | 2.66  |                      |
| Pentanol                               | 0.0068 | 5                      | 25.0                          | 4.26  | (3.038) <sup>c</sup> |
|  | 0.0074 | 8                      | 18.4                          | 6.58  |                      |
|  | 0.0080 | 5                      | 14.0                          | 4.00  |                      |
|  | 0.0092 | 6                      | 10.6                          | 2.16  |                      |
|  | 0.0105 | 10                     | 10.5                          | 2.19  |                      |

$$\frac{100}{\text{Mean}} \times \sqrt{\frac{\sum d^2}{n(n-1)}}$$

while  $A$  is a constant for each alcohol, decreasing in value as the number of carbon atoms increases. From equations (1) and (2), it is apparent that

$$a = \frac{A_1}{A_2} = \frac{A_2}{A_3} = \frac{A_3}{A_4} = \frac{A_4}{A_5}$$

From the plots in Figs. 2 and 3 the values of  $B$  for the frog and *Planaria* were found to be 4.50 and 2.80 respectively. By substituting

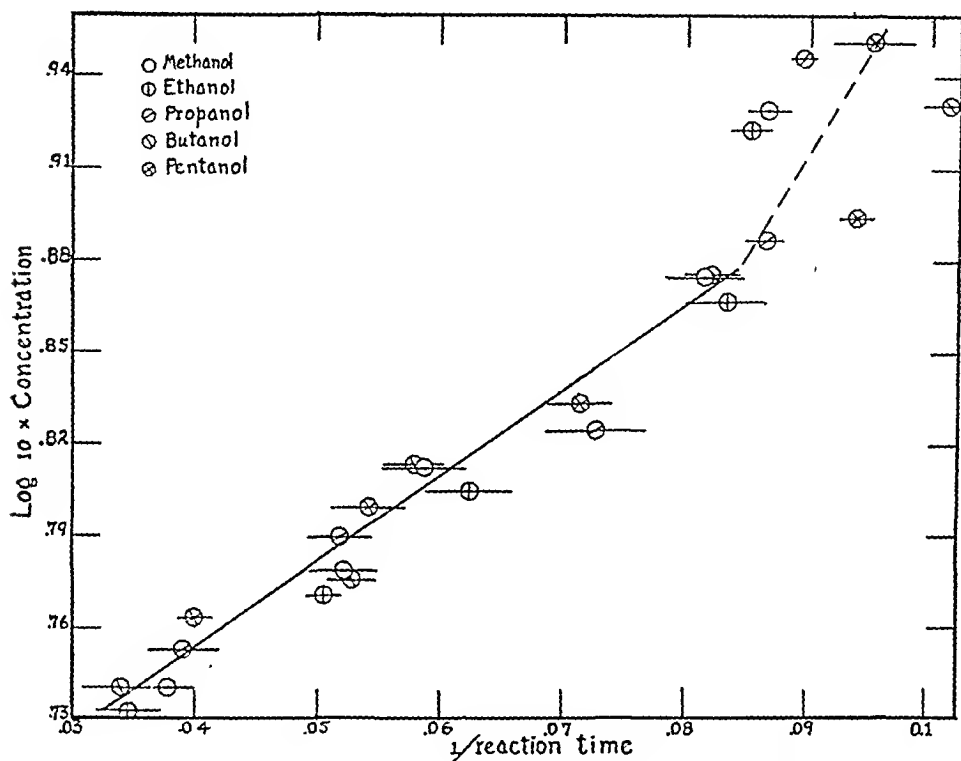


FIG. 3. Plot of reciprocal of reaction time of *Planaria* to five alcohols against the logarithm of  $10 \times$  concentration. The length of the horizontal lines through the circles represents the magnitude of the mean error of the mean expressed as percentage of the mean. The concentrations have been multiplied by the respective ordinate factors given in Column 5 of Table IV. The equation of the line is  $B = t \log \frac{C}{A}$  where the slope,  $B$ , = 2.8 and  $A$ , the antilog of the Y-intercept, = 0.4376.

them in equation (2),  $A$  for each alcohol and each animal was then calculated from the data, and the ratios between successive  $A$ 's were determined. These values, as presented in Table V, indicate that the ratio 3 is correct for *Planaria*, but slightly too high for the frog. Such

a difference between animals may be expected since there is no *a priori* reason why the ratio for stimulating efficiency of successive members of a homologous series should be the same for all animals.

It has been assumed that the "rate of the reaction" measured in our studies is proportional to the amount of energy change at the receptive surface, and that this change is determined by the non-polar portion of the molecule, or by the length of the carbon chain. A generalized theory to account for energy changes at interfaces on the basis of a comparison between the activities of the more active and the less active portions of polar organic molecules has been developed by Langmuir (1917, 1925, 1926, 1929) and by Harkins (1924, 1926).

TABLE V

Values of  $A$  in equation (2) for the frog and for *Planaria*, when  $B = 4.5$  and 2.8 respectively.

| Alcohol       | Frog   |                   | <i>Planaria</i> |                   |
|---------------|--------|-------------------|-----------------|-------------------|
|               | $A$    | $\frac{A_1}{A_2}$ | $A$             | $\frac{A_1}{A_2}$ |
| Methanol..... | 1.0380 | 2.84              | 0.4359          | 3.008             |
| Ethanol.....  | 0.3655 | 2.97              | 0.1449          | 3.091             |
| Propanol..... | 0.1229 | 2.84              | 0.0468          | 2.898             |
| Butanol.....  | 0.0432 | 2.98              | 0.0161          | 3.157             |
| Pentanol..... | 0.0145 | Ave. 2.90         | 0.0051          | Ave. 3.038        |

According to Langmuir's "principle of independent surface action," it is concluded that the "forces acting between molecules of various types" among a large class of substances are "dependent mainly on the nature of the surfaces of the molecules which are in contact." For example, in an air-water interface organic molecules orient in such a way that the polar groups are directed towards the water and the less active groups away from the water. At a liquid-liquid interface molecules of an organic solute will be oriented according to the attraction or repulsion of each liquid for certain groups in the molecules of the solute. At an interface between an organic liquid and water the polar groups of the solute molecules will be directed towards the water and the non-polar parts will be drawn towards the organic

liquid. At a receptive surface in animals, therefore, the particular orientation of a polar organic molecule which is stimulatory will determine the nature of the stimulation process. In a homologous series of polar organic compounds the efficiency for stimulating the receptor will be determined either by the potential of the polar groups, or by the structure and magnitude of the non-polar groups, whenever one or the other becomes dominant in its effect. A similar interpretation has been made by Crozier (1918) in an illuminating discussion of the factors involved in stimulation by fatty acids.

Hixon and Johns (1927) have arranged organic radicals in an activity series based upon the variations of the polar properties of compounds in any series, such as  $R-OH$ ,  $R-COOH$ ,  $R-NH_2$ , etc. A similar series could also be arranged for the stimulating efficiency of its members if the effect produced were solely a function of the potential of the polar group. For example, if the stimulating efficiency depends upon the hydrogen ion concentration resulting from the dissociation of the carboxyl group, then the radical attached to that group would exert its effect only through the changes it might produce on the dissociation constant. Crozier (1916) has clearly shown that in certain acid series, where the dissociation constants are high, the penetration efficiency of members of the series through a living membrane is chiefly a function of the hydrogen ion concentration. However, it is to be expected that the anion and any undissociated molecules would also exert some effect especially in stimulation where changes in surface energy may be a dominant factor. From a similar study made on the stimulating efficiency of acids, Crozier (1916) states: "The strong acids follow the order of their ionization, but this at such high dilutions that the correspondence probably has little real reference to dissociation." Warburg (1921) has studied the effect of  $N/100$  solutions of acetic aldehyde, propionic aldehyde, butyric aldehyde and valeric aldehyde on the respiration of blood cells. He found that equal concentrations of these compounds were necessary to reduce respiration by 50 per cent. He concludes that the hindrance to respiration is caused by the specific aldehyde group. If the potential of the polar group of the aliphatic aldehydes used in Warburg's experiments is relatively constant, and if the effect produced by these compounds is primarily due to a reaction of the aldehyde group with some vital constituent in the

cells, equivalent concentrations should produce similar results. Although Warburg's work on respiration is not strictly analogous to the results presented in this paper, it agrees with the interpretation of studies on stimulation.

If the potential of the polar group in any series is relatively constant and primarily effective through its influence upon the orientation of a member of a series at the receptor surface, then the stimulating efficiency of that member may be related to the structure of the less active portion of the molecule. Langmuir (1917) has calculated the loss in potential energy,  $\lambda$ , when a gram molecule of an organic substance passes from the interior to the surface film formed between air and water, from the equation

$$\lambda_{cal} = \lambda_p + 625 n$$

where  $\lambda_p$  has a different value for each type of compound, and  $n$  equals the number of carbon atoms. In other words, in any homologous series the effect of the addition of each  $\text{CH}_2$  group upon  $\lambda_p$  is constant. Thus in the normal aliphatic alcohols, ester or fatty acids series, where the potential of the polar group is almost constant, the degree of stimulation should increase as the length of the carbon chain increases, provided the more active portion of the molecule functions primarily as an orienting group. Crozier (1916) has shown that when a low hydrogen ion concentration is approached, as in the monobasic fatty acid series, the dominant factor in determining the degree of stimulation is the length of the carbon chain. To this rule formic acid was an exception. In his discussion of the acid penetration series, Crozier pointed out that formic acid has a higher dissociation constant than its homologues. While discussing the effect of additional  $\text{CH}_2$  groups upon the field of force around a carboxyl group in the fatty acid series, Langmuir (1929) states: "The forces near the surface of the carboxyl group should be practically constant for all acids higher than propionic acid, and would be roughly the same in the case of acetic acid, but might be considerably different in the case of formic acid." According to Hixon and Johns (1927), this variation might also be expressed in terms of  $\lambda$ , since "if the orientation of the molecules is a function of the polar group,  $\Delta\lambda$  for any homologous series would not be constant

unless the potential of this group remained unchanged as  $\text{CH}_2$  units were interposed." If a relatively slight change in the potential of the polar group may produce an added stimulating effect it might be expected that only where  $\Delta\lambda$  was actually a constant would the order of stimulating efficiency be determined by the number of intervening  $\text{CH}_2$  units in the carbon chain of any homologous series. On the other hand, if such a change in the potential of a polar group in any homologous series is primarily effective for stimulation through its influence upon orientation of the molecules in a surface, the first member of the fatty acid series would probably not be much of an exception to the rule. This statement is based upon Langmuir's interpretation of the effect of the fatty acids upon the surface tension of water, where formic acid is not considered an exception. For example, Loeb (1908-09) found the following order of efficiency for the normal fatty acids in membrane formation in the sea urchin egg:

butyric > propionic > acetic > formic

Formic acid is here less effective than acetic, so it may be assumed that membrane formation by fatty acids, including formic acid, is primarily due to a change in surface energy induced by the orientation of the molecules of the members of that series at the surface of the egg.

In the aliphatic alcohol series, the potential of the polar group in the different molecules remains practically constant throughout, and is primarily effective for stimulation through its influence upon surface orientation. It would be expected therefore that the degree of stimulation by successive alcohols would reveal a definite relationship to the structure and magnitude of the non-polar groups in the molecules. This relationship is exponential for the barnacle, the frog, and *Planaria*, and may be mathematically expressed. The formula derived contains two constants, one of which is characteristic of the process of stimulation by alcohol in any one animal. The other constant characterizes each alcohol, the ratio of the values from successive alcohols being identical with the ratio of successive concentrations necessary to produce a given effect. Although the exact nature of stimulation by alcohols is not yet revealed, there is provided an experimental and theoretical foundation upon which further studies of homologous

series may be based. It is hoped that progress can thus be made towards an analysis of the initial energy changes involved in chemical stimulation.

#### SUMMARY

1. The stimulating efficiencies of some normal primary aliphatic alcohols have been determined for the barnacle, the frog, and *Planaria*, under conditions which do not involve narcosis or simultaneous stimulation by other agents.

2. Concentrations of the successive alcohols necessary to produce a given stimulatory effect vary according to the following geometrical series:  $1: a^{-1}: a^{-2}: a^{-3}: a^{-4}: \dots$ , where  $a$  represents some real number.

3. Within certain limits the relationship between the logarithm of the concentration necessary to produce a given effect and the reciprocal of the reaction time is linear in the frog and in *Planaria*.

4. The concentration effect may be expressed by an equation which contains one constant characteristic of the alcohol series, and another one characteristic of each member. The ratio of the latter constants for successive alcohols represents  $a$  in the above series.

5. The stimulation by alcohols in these animals is considered to be due to energy changes at the receptive surfaces, brought about by a definite orientation of the respective alcohol molecules. Increase in stimulating efficiency as the number of  $\text{CH}_2$  groups increase must be due to the rôle of the non-polar portion of the alcohol molecule, since the polar group remains practically constant throughout the series.

6. In homologous series of organic compounds it is conceived that stimulating effects will be produced either by the polar group or the non-polar group, according to which one becomes dominant in effect, or to a combination of the two.

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# THE ARGININE AND PREARGININE GROUPS IN EDESTIN

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## I

### INTRODUCTION

Evidence was given in 1928<sup>1</sup> to show that the combination of strong acid or alkali with a protein is produced by forming salts with the "extra groups" of those trivalent amino acids which can be isolated from the protein (namely, aspartic acid, glutamic acid, tyrosine, histidine, lysine, and arginine). The "titration curve" of a protein corresponds approximately to the amounts of these various groups, each with its typical titration index.<sup>2</sup>

Arginine, however, formed a marked exception. It appeared to contribute to the titration curve in much smaller amount than is found on hydrolysis. On the other hand, a group of unknown origin having a titration index of pH 4.6 was present in amounts equal to the deficiency in arginine.

The name *prearginine* was given to this group on the assumption that it is a chemical group which ionizes at pH 4.6 in proteins but yields *arginine* on hydrolysis of the protein.

It has subsequently developed that the index 8.1 assumed for the "extra group" of *arginine* was incorrect (being based on data obtained

<sup>1</sup> Simms, H. S., *J. Gen. Physiol.*, 1928, 11, 629; 1928, 12, 231.

<sup>2</sup> A "dissociation index" is:  $pK' = -\log K'$  where  $K'$  is the apparent dissociation constant of a weak electrolyte (referred to the hydrogen ion). The dissociation index represents the pH value at which the group is half ionized.

The "titration indices" ( $pG'$ ) determine the titration curve and cannot be correctly called "dissociation indices" although there is little numerical difference between the titration constants ( $G'$ ) and the dissociation constants ( $K'$ ). The use of these two sets of constants and indices is confusing but unavoidable. See Simms, H. S., *J. Am. Chem. Soc.*, 1926, 48, 1239.

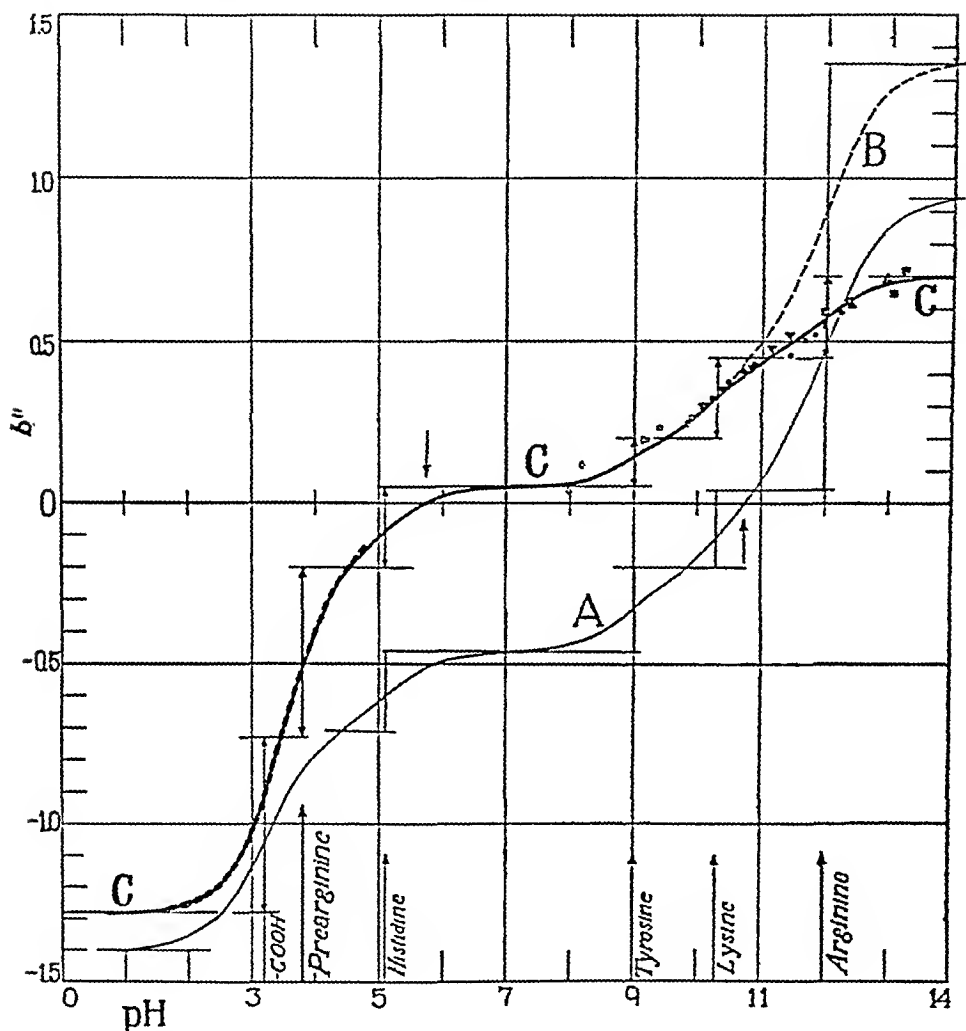


FIG. 1. Titration curve of edestin. The solid symbols represent data from clear solutions; the open symbols, from cloudy solutions. The heavy dash line is drawn to fit the data in acid solution of Hitchcock and of Kodama.

Curve A is calculated from the amino acids found on hydrolysis.

Curve B (which branches off from C) represents the way edestin would ionize if it had the above groups (found on hydrolysis), but in addition had an *extra* group at pH 3.8.

Curve C corresponds with the data in Column 5 of Table I.

from impure material). Schmidt<sup>2</sup> has obtained the value  $\text{pK}'_2 = 12.5$  which agrees with that of Hunter and Borsook ( $\text{pK}'_2 = 13.85$  at  $18^\circ\text{C}$ .).

<sup>2</sup> Schmidt, L. A., Kirk, P. L., and Appleman, W. K., *J. Biol. Chem.*, 1930, 88,

We have repeated our titration of arginine and corroborate the value of Schmidt.

In order to determine whether this would alter the above theory it was necessary to obtain more accurate data than were available, on a protein in very alkaline solution. Edestin was again chosen due to its high content in arginine.

## II

## RESULTS

A number of experiments were performed with edestin in solutions up to 14 per cent and at pH values up to 13.5. A method was adopted which as far as possible caused the unavoidable errors to cancel out. The data are given in Fig. 1 (and Table II).

An analysis of these data is given in Table I.

TABLE I

| (1)                              | (2)                | (3)                | (4)                      | (5)            | (6)        |
|----------------------------------|--------------------|--------------------|--------------------------|----------------|------------|
| Type                             | Groups             | Indices in edestin | Equivalents per 1000 gm. |                |            |
|                                  |                    |                    | On hydrolysis            | Titration data | Difference |
| Acidic                           | Dicarboxylic acids | $pG_1' = 3.2$      | 0.72                     | 0.55           | -0.14      |
|                                  | Tyrosine           | $pG_4' = 9.0$      | .25                      | .15            | -.10       |
|                                  | Sum                |                    | 0.97                     | 0.70           |            |
| Basic                            | PREARGININE        | $pG_2' = 3.8$      | 0                        | 0.53           | +0.53      |
|                                  | Histidine          | $pG_3' = 5.1$      | 0.25                     | .25            | 0          |
|                                  | Lysine             | $pG_5' = 10.3$     | .25                      | .25            | 0          |
|                                  | ARGININE           | $pG_6' = 12.0$     | .90                      | .25            | -0.65      |
|                                  | Sum                |                    | 1.40                     | 1.28           |            |
| Sum of prearginine plus arginine |                    |                    | 0.90                     | 0.78           |            |

Values in Column 4 are those listed by Mitchell and Hamilton,<sup>4</sup> except for histidine and lysine in which case the values of Van Slyke<sup>5</sup> are taken. The figure for dicarboxylic acids represents aspartic acid (0.76) plus glutamic acid (1.31) minus ammonia (1.35) and represents the carboxyl groups not bound by ammonia.

<sup>4</sup> Mitchell and Hamilton, *The Biochemistry of the Amino Acids*, Chem. Cat. Co., 1929, 191.

<sup>5</sup> Van Slyke, D. D., *J. Biol. Chem.*, 1911, 10, 15.

If edestin contained the free groups found on hydrolysis (Table I, Column 4) it would have a titration curve represented by Curve *A* in Fig. 1. This is obviously not the case.

If edestin had approximately the above free groups, and *in addition* a group with an index at pH 3.8, in proper amount, the titration curve would be that found experimentally up to pH 10.5; but above that it would have Curve *B*. This is also not the case.

The experimental data agree well with Curve *C* which corresponds to the values in Column 5 of Table I. *This supports the theory that the basic group in proteins having a titration index at pH 3.8 to 4.6 (depending on the protein) is due to a group which yields arginine on hydrolysis and may be properly called prearginine.*

### III

#### EXPERIMENTAL

The two experiments, recorded in Tables II and III, were performed in water-jacketed hydrogen electrode cells<sup>6</sup> with a saturated KCl liquid junction. A separate solution was made up, from the mother solution, for each determination. The cells were standardized against 0.100 M HCl (pH 1.075).

The four experiments in Tables IV to VII were performed with considerably higher concentrations of edestin (as high as 14 per cent) and it was necessary to use a titrating electrode with agar-KCl junction. In each experiment all the readings were taken on a single solution to which molar NaOH (or HCl) was added with a weighing burette.

Owing to the large correction for hydroxyl ion concentration to be made above pH 13, it was not found satisfactory to standardize the electrodes against HCl. Hence the electrodes were standardized against 0.05 M NaOH before (and sometimes after) each experiment. In Fig. 2 are the values of  $\text{pH} - \log oh$  for NaOH<sup>7</sup> as determined with the water-jacketed (liquid junction) cells. By using this chart as a standard of  $\text{pH} - \log oh$  in correcting the edestin data, any errors due to the standard cell, the sodium hydroxide solution or the condition of the electrodes, would be eliminated in the calculation of  $\frac{h - oh}{c}$ .

<sup>6</sup> Simms, H. S., *J. Am. Chem. Soc.*, 1923, 45, 2503.

<sup>7</sup> *oh* represents hydroxyl ion concentration in moles per liter. The value of  $\text{pH} - \log oh$  is equal to  $\text{pK}_w + \log f_{OH}$  where  $f_{OH}$  is the activity coefficient of the hydroxyl ion (namely,  $f_{OH} \times oh = OH = \text{hydroxyl ion activity}$ ).

This method does assume, however, that solutions of the same ionic strength have the same liquid function potential and dielectric constant. The ionic strength of the protein solutions will be discussed below.

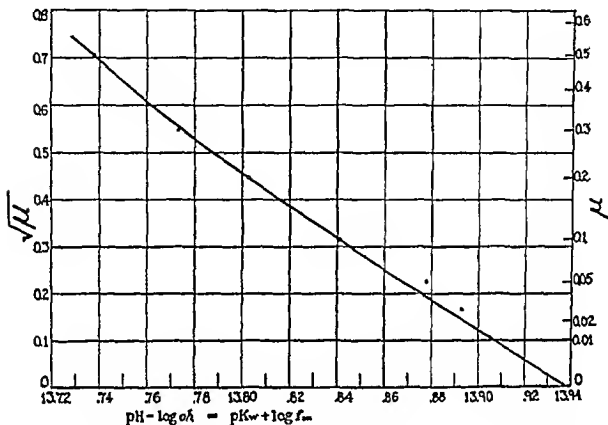


FIG. 2. Data of the titration of NaOH. Assuming complete dissociation, the concentration of hydroxyl ion ( $a_h$ ) is taken equal to the molarity of NaOH.<sup>7</sup>

#### IV

##### Calculations

##### A. Equivalents of Combined Base

The data in Tables II to VII were calculated as follows:

(1) The volumes of *strong base* ( $V_b$ ) or *strong acid* ( $V_a$ ) were calculated from the weight of the standard molar solutions, using the density at 25°C.

(2) The volume of *solution* ( $V$ ) was taken as the sum of the volumes of water plus strong base (plus strong acid when present) and represents *protein-free* volume.

(3) The number of *millimoles* of strong base *minus* acid is:

$$1000 (B - A) = 1000 (V_B M_B - V_A M_A) \quad (1)$$

where  $M_B$  and  $M_A$  are the moles per liter in the standard base and acid solutions.

(4) The number of millimoles *per liter* is:

$$b - a = \frac{1000 (B - A)}{V} \quad (2)$$

(5) The *concentration* in moles per liter of a substance of molecular weight  $M$  is:

$$c = \frac{1000 W}{M V} \quad (3)$$

where  $W$  is the weight. But in dealing with a protein or any other substance of uncertain molecular weight it is more convenient to substitute 1000 for the molecular weight (giving us a final value of equivalents per 1000 gm., or milli-equivalents per gram):

$$c' = \frac{W}{V} \text{ gm. per ml.} = \frac{c M}{1000} \quad (3a)$$

(6) The number of *equivalents* of strong base minus acid is:

$$\frac{b - a}{c} = \frac{M (B - A)}{W} \text{ equivalents added per mole} \quad (4)$$

Or, for a protein:

$$\frac{b - a}{c'} = \frac{1000 (B - A)}{W'} \text{ equivalents added per 1000 gm.} \quad (4a)$$

(7) The ionic strength ( $\mu$ ) was calculated as explained below and used with the curve in Fig. 2 to obtain the value of  $\text{pH} - \log oh$ . The experimental pH value was subtracted from this and the value of  $\frac{h - oh}{c'}$  was calculated (where  $h$  may be neglected in alkaline solutions).

(8) The "corrected equivalents of base," or the "combined base," is:

$$b' = \frac{b - a}{c} + \frac{h - oh}{c} \text{ equivalents combined per mole} \quad (5)$$

Or, for a protein:

$$b'' = \frac{b-a}{c'} + \frac{h-oh}{c'} \text{ equivalents combined per 1000 gm.} \quad (5a)$$

$$b'' = b' \text{ when } M = 1000$$

### B. Calculation of Ionic Strength

If we take the experimental data for the effect of ionic strength on protein ionization and assume a point charge, we can calculate an "apparent valence" ( $\nu_A$ ) which is not much greater than unity.<sup>8</sup> This is because the charges on the ionized groups in a protein molecule are so distant from each other that they act almost like separate monovalent ions (*i.e.*, *separate* point charges). The value  $\nu_A = 1.8$  was found in acid solutions of gelatin, and  $\nu_A = 2.4$  in alkaline solutions.

By using the same assumptions (of point charge) we can use this apparent valence to calculate the approximate contribution of the protein to the ionic strength of its solution. In alkaline solution the contribution of the protein and its bound cation will be:

$$\mu_0 = \frac{\nu_A^2 + \nu_A}{2 \nu_A} b'' c' = \frac{\nu_A + 1}{2} b'' c' = 1.7 b'' c'$$

while the contribution of the unbound NaOH will be given by the hydroxyl ion *concentration*, hence:

$$\begin{aligned} \mu &= 1.7 b'' c' + oh + a \\ &= 1.7 (b - a - oh) + oh + a \\ &= 1.7 b - 0.7 oh - 0.7 a \end{aligned}$$

The data of edestin were calculated in this manner and confirmed the findings with gelatin that the contribution of protein to the ionic strength is even less than that given by this approximation. The results in Tables II to VII and Fig. 1 were calculated by assuming  $\nu_A = 1$  (*i.e.*, infinitely distant point charges), hence:

$$\mu = b'' c' + oh + a = b$$

(where the amount of strong base  $b$ , being greater than the strong acid  $a$ , includes all free inorganic salt which may be present).

<sup>8</sup> Simms, H. S., *J. Gen. Physiol.*, 1928, 11, 613.



TABLE II

Titration of edestin in jacketed electrodes with saturated KCl liquid junction at 25°C. (pH standard: 0.100 M HCl has pH 1.075).

Each solution contained 0.025 gm. edestin, plus 0.015 millimole of NaOH, plus the indicated volume of 0.0200 M HCl and was made up to 10.0 ml.  $c' = 0.0025$  and  $\mu = 0.0015$ . All the solutions were cloudy.

| $V_A$ | pH    | $\frac{b-a}{c'}$ | $b''$ |
|-------|-------|------------------|-------|
| 0.40  | 9.848 | 0.280            | 0.243 |
| .50   | 9.135 | .200             | .193  |
| .60   | 8.181 | .120             | .119  |
| .70   | 7.993 | .040             | .039  |

TABLE III

Titration of edestin in jacketed electrodes with saturated KCl liquid junction at 25°C. (pH standard: 0.100 M HCl has pH = 1.075).

Each solution contained 0.0625 gm. edestin plus 0.0375 millimole of NaOH, plus the indicated volume of 0.0200 M HCl and was made up to 10.0 ml.  $c' = 0.00625$  and  $\mu = 0.00375$  in each solution.

| $V_A$ | pH     | $\frac{b-a}{c'}$ | $b''$ |
|-------|--------|------------------|-------|
| 0     | 10.886 | 0.600            | 0.431 |
| 0.25  | 10.710 | .520             | .408  |
| .50   | 10.482 | .440             | .374  |
| .75   | 10.208 | .360             | .325  |
| .87   | 10.078 | .320             | .294  |
| 1.00  | 9.898  | .280             | .263* |
| 1.12  | 9.396  | .240             | .235* |
| 1.25  | 9.195  | .200             | .196* |

\* Cloudy.

TABLE IV

Titration of edestin in a titrating electrode with agar-KCl bridge at 25°C. (pH standard: 0.0494 M NaOH has pH = 12.562).

4.996 gm. (*W*) of edestin in an initial non-protein volume of 35.00 ml. containing 2.420 millimole NaOH. The amount of 0.991 M NaOH is indicated by  $V_B$  (determined by weight;  $d = 1.040$  at 25°C.).

| $V_B$ | $c' = \frac{W}{V}$ | $\mu$ | pH     | $\frac{b-a}{c'}$ | $\delta''$ |
|-------|--------------------|-------|--------|------------------|------------|
| 2.442 | 0.1428             | 0.069 | 11.438 | 0.484            | 0.457      |
| 2.774 | .1414              | .078  | 11.672 | .550             | .503       |
| 2.960 | .1407              | .083  | 11.807 | .587             | .523       |
| 3.246 | .1396              | .090  | 11.979 | .644             | .547       |
| 3.859 | .1372              | .105  | 12.216 | .765             | .591       |
| 4.457 | .1350              | .119  | 12.372 | .884             | .627       |

TABLE V

Titration of edestin in a titrating electrode with agar-KCl bridge at 25°C. (pH standard: 0.0494 M NaOH has pH = 12.562).

2.500 gm. (*W*) of edestin in an initial non-protein volume of 17.72 ml. containing 1.708 millimole NaOH. The volume of 0.991 M NaOH is indicated by  $V_B$  (determined by weight;  $d = 1.040$  at 25°C.).

| $V_B$ | $c' = \frac{W}{V}$ | $\mu$  | pH     | $\frac{b-a}{c'}$ | $\delta''$ |
|-------|--------------------|--------|--------|------------------|------------|
| 1.724 | 0.1411             | 0.0964 | 11.966 | 0.683            | 0.569      |
| 5.523 | .1161              | .254   | 13.040 | 2.19             | 0.65       |
| 8.619 | .1015              | .346   | 13.217 | 3.41             | .72        |

TABLE VI

Titration of edestin in a titrating electrode with agar-KCl bridge at 25°C. (pH standard: 0.0494 M NaOH has pH = 12.562).

2.500 gm. (*W*) of edestin in an initial non-protein volume of 18.14 ml. containing 2.168 millimole NaOH. The volume of 0.991 M NaOH is indicated by  $V_B$  (determined by weight;  $d = 1.040$  at 25°C.).

| $V_B$ | $c' = \frac{W}{V}$ | $\mu$ | pH     | $\frac{b-a}{c'}$ | $b''$ |
|-------|--------------------|-------|--------|------------------|-------|
| 2.188 | 0.1371             | 0.109 | 12.376 | 0.867            | 0.615 |
| 4.583 | .1218              | .221  | 12.936 | 1.817            | .692  |

TABLE VII

Titration of edestin in a titrating electrode with agar-KCl bridge at 25°C. (pH standard: 0.0492 M NaOH has pH = 12.560).

0.600 gm. (*W*) of edestin in an initial non-protein volume of 30.45 ml. containing 0.413 millimole NaOH. To this was added 0.991 M HCl in volumes indicated by  $V_A$  (determined by weight;  $d = 1.016$  at 25°C.).

| $V_A$ | $c' = \frac{W}{V}$ | $\mu$  | pH     | $\frac{b-a}{c'}$ | $b''$ |
|-------|--------------------|--------|--------|------------------|-------|
| 0     | 0.01971            | 0.0136 | 11.423 | 0.688            | 0.518 |
| 0.072 | .01967             | .0159  | 11.144 | .570             | .481  |
| .139  | .01962             | .0180  | 10.841 | .458             | .414  |
| .196  | .01957             | .0198  | 10.383 | .365             | .349  |
| .230  | .01956             | .0209  | 10.083 | .308             | .300* |

\* The last solution was cloudy. The other solutions were clear.

## V

## SUMMARY

The author corroborates the data of Schmidt showing that the dissociation index of the third group of arginine is  $pK_3' \approx 12.5$ .

New titration data of edestin have been obtained in very alkaline solutions and show that there is a corresponding group with a titration index of  $pG' = 12.0$ , but present in much less quantity than can account for the arginine found on hydrolysis. The data support the theory that the combination of strong base or strong acid with proteins is produced by the formation of salts with the "extra groups" of those trivalent amino acids which can be isolated from the protein, with the exception of arginine. Arginine contributes to the titration curve in much smaller amount than is found on hydrolysis. This deficiency in the arginine group may be accounted for by the basic group in proteins having a titration index of  $pG' = 3.8$  to  $4.6$  (depending on the protein), which apparently yields arginine on hydrolysis, and may properly be called *prearginine*.



# THE COMBINATION OF EDESTIN WITH HYDROCHLORIC ACID

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In a recent paper<sup>1</sup> it was shown that the amounts of the ions of HCl combined with gelatin could be calculated from electromotive force measurements of cells without liquid junction, of the type Ag, AgCl, HCl + protein, H<sub>2</sub>. The maximum combining capacity of gelatin for H<sup>+</sup>, as calculated by this method, agreed with values obtained in other ways. The present paper records experiments in which this method has been applied to the protein edestin.

## EXPERIMENTS<sup>2</sup>

A sample of edestin had been prepared from ground hemp seed by Osborne's method,<sup>3</sup> slightly modified.<sup>4</sup> It was freed from electrolytes by dialysis against running distilled water in a rocking apparatus of the type described by Kunitz and Simms.<sup>5</sup> The liquid obtained by centrifuging the suspension after dialysis had a specific conductivity of  $4.4 \times 10^{-6}$  reciprocal ohms at 30°C., while the corresponding value for the water used was  $2.6 \times 10^{-6}$ . Solutions were prepared by mixing portions of the suspension with measured amounts of standard hydrochloric acid, made up from a distilled constant boiling mixture and redistilled water, and checked volumetrically by Na<sub>2</sub>CO<sub>3</sub> and gravimetrically by AgCl. The concentrations of acid and of edestin were determined by weighing the whole solution containing a known volume of standard acid, neutralizing a weighed portion to methyl red with NaOH, and drying this neutralized portion to constant weight at 110°C. The weight of edestin was obtained by subtracting the weight of NaCl (calculated from the original HCl content of the solution) from the observed dry weight. The weight of water in a given weight of solution was obtained by sub-

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<sup>1</sup> Hitchcock, D. I., *J. Gen. Physiol.*, 1928-29, 12, 495.

<sup>2</sup> Most of the measurements were made by Esther R. Mason. The edestin was prepared by C. E. Heinrichs.

<sup>3</sup> Osborne, T. B., *J. Am. Chem. Soc.*, 1902, 24, 28.

<sup>4</sup> Hitchcock, D. I., *J. Gen. Physiol.*, 1921-22, 4, 597.

<sup>5</sup> Kunitz, M., and Simms, H. S., *J. Gen. Physiol.*, 1927-28, 11, 641.

tracting the sum of the weights of edestin and HCl from the total weight. The concentrations were expressed as molality of HCl and grams of protein per 1000 gm. H<sub>2</sub>O. The measurements of E.M.F. were carried out at 30°C., as described in the previous paper.<sup>1</sup> The data are given in the first 3 columns of Table I. Each figure for the E.M.F. represents the mean of results with 3 or 4 cells not differing by more than 0.0003 volt, and each constant to  $\pm 0.0001$  volt for at least 1 hour.

TABLE I

*Electromotive Force at 30°C. of the Cells*

Ag, AgCl, HCl, H<sub>2</sub> and Ag, AgCl, HCl + edestin, H<sub>2</sub>

| <i>m</i> | <i>g</i> | <i>E</i><br>(observed) | <i>E'</i> <sub>0</sub> | <i>E</i><br>(calculated) | $\Delta E$ | pH<br>(approx.) |
|----------|----------|------------------------|------------------------|--------------------------|------------|-----------------|
| 0.0100   | 0        | 0.4650                 | 0.2244                 |                          |            |                 |
| 0.0200   | 0        | 0.4303                 | 0.2260                 |                          |            |                 |
| 0.0301   | 0        | 0.4101                 | 0.2271                 |                          |            |                 |
| 0.0401   | 0        | 0.3958                 | 0.2278                 |                          |            |                 |
| 0.0501   | 0        | 0.3848                 | 0.2284                 |                          |            |                 |
| 0.1004   | 0        | 0.3510                 | 0.2309                 |                          |            |                 |
| 0.1007   | 13.05    | 0.3568                 | 0.2307                 | 0.3570                   | +0.0002    | 1.08            |
| 0.1020   | 18.55    | 0.3592                 | 0.2307                 | 0.3592                   | 0          | 1.11            |
| 0.1050   | 42.35    | 0.3732                 | 0.2304                 | 0.3730                   | -0.0002    | 1.32            |
| 0.1055   | 62.70    | 0.3961                 | 0.2301                 | 0.3961                   | 0          | 1.67            |

*m* = moles HCl per 1000 gm. H<sub>2</sub>O.

*g* = gm. protein per 1000 gm. H<sub>2</sub>O.

*E* (observed) = E.M.F. in volts, corrected to 1 atmosphere dry H<sub>2</sub>.

*E'*<sub>0</sub> =  $E + 0.1203 \log m$ , for cells containing HCl alone.

*E* (calculated) =  $E'_{0} - 0.06015 \log (m - gx)(m - gy)$ .

*x* = moles H<sup>+</sup> combined with 1 gm. edestin =  $13.4 \times 10^{-4}$ .

*y* = moles Cl<sup>-</sup> combined with 1 gm. edestin =  $3.9 \times 10^{-4}$ .

$\Delta E$  = *E* (calculated) - *E* (observed).

pH (approx.) =  $-\log (m - gx)$ .

### *Interpretation of Results*

The agreement of the values in the third and fifth columns of Table I indicates that the data may be explained by assigning to 1 gm. of edestin a combining capacity of  $13.4 \times 10^{-4}$  equivalents of H<sup>+</sup> and  $3.9 \times 10^{-4}$  equivalents of Cl<sup>-</sup>, both figures being constant in the range of these experiments, from about pH 1.1 to 1.7. The assumptions used in arriving at these values, and the method of calculation, are as fol-

lows. The relation between E.M.F. and ionic activity is given by the thermodynamic equation,

$$E = E_o - 0.06015 \log m_H m_{Cl} \gamma^2, \quad (1)$$

where  $E$  is the observed E.M.F. in volts,  $E_o$  is a constant,  $m_H$  and  $m_{Cl}$  are the molalities of the ions, and  $\gamma$  is the geometric mean activity coefficient of  $H^+$  and  $Cl^-$ . Letting  $E'_o = E_o - 0.1203 \log \gamma$ , this becomes  $E = E'_o - 0.06015 \log m_H m_{Cl}$ , or

$$E = E'_o - 0.06015 \log (m - gx) (m - gy). \quad (2)$$

Here  $m$  is the total molality of  $HCl$ ,  $x$  and  $y$  are the numbers of moles of  $H^+$  and  $Cl^-$ , respectively, combined with 1 gm. of protein, and  $g$  is the number of grams of protein per 1000 gm.  $H_2O$  in the solution. As a first approximation, the value of  $E'_o$  for the protein cells was assumed to be constant and equal to that calculated for the cell containing 0.1004  $M$   $HCl$  without protein by the relation

$$E'_o = E + 0.1203 \log m, \quad (3)$$

which follows from equation (1) and the definition of  $E'_o$ . When the equations obtained by substituting the measured values of  $E$ ,  $m$ , and  $g$  in equation (2) were solved simultaneously for  $x$  and  $y$  by the graphical method of the previous paper,<sup>1</sup> the curves did not intersect in a single point, but the provisional values  $x = 13.4 \times 10^{-4}$  and  $y = 3.5 \times 10^{-4}$  were obtained by averaging the different intersections. This relatively high value for  $y$  indicated a possible error in the assumption that  $E'_o$  (or  $\gamma$ ) was constant for all the solutions. If all the protein is combined with  $H^+$ , and a certain amount also combined with  $Cl^-$ , then the latter part should behave as un-ionized molecules. Hence the ionic strength of such a solution should not be equal to  $m$ , but to  $m - gy$ . Provisional values of  $m - gy$  were calculated and values for  $E'_o$  corresponding to them were interpolated from a plot of the  $E'_o$  values given for pure  $HCl$  in the first part of Table I. These values for  $E'_o$  are given in the last part of Table I, and were used in re-calculating the edestin data. A second application of the graphical method again failed to give a single intersection of the curves for  $y$  as a function of  $x$ , but the values selected from this plot,  $x = 13.4 \times 10^{-4}$  and  $y = 3.9$



$\times 10^{-4}$ , gave such agreement of the observed and calculated values for  $E$  that further repetitions of the procedure seemed unnecessary. The second approximation reduced the average deviation of the calculated values (average of  $\Delta E$ ) only from 0.00013 to 0.00010 volt.<sup>6</sup>

#### DISCUSSION

The value here obtained for the combining capacity of edestin for  $H^+$  is slightly higher than any of the values compiled by Cohn,<sup>7</sup> who selected  $12.7 \times 10^{-4}$  moles HCl per gram edestin as the probable maximum value. In an earlier paper<sup>4</sup> curves were given by the writer which lead to the value  $12.7 (\pm 0.6) \times 10^{-4}$  equivalents of HCl or  $H_2SO_4$ , and the same number of moles of  $(COOH)_2$  or  $H_3PO_4$ , as the combining capacity of 1 gm. of edestin at pH 2.0 or below. Somewhat later<sup>5</sup> another curve was obtained for HCl which points to the value  $14.7 \times 10^{-4}$ . These divergent values are probably to be explained by insufficient removal of combined acid or base from some of the protein preparations. Attempts to purify edestin are handicapped by the uncertainty which still exists as to the exact location of its isoelectric point. It should be added that, according to Osborne,<sup>3</sup> edestin is changed by acid solutions into a substance insoluble in NaCl solutions. Hence any value for the maximum combining capacity with acids is probably a property of this denatured substance edestan rather than of the native protein edestin.

The present value, like all previous values obtained from experiments with edestin in solution in hydrochloric acid, is much lower than that recently reported by Bancroft and Barnett<sup>9</sup> for the combining capacity of dry edestin with hydrogen chloride gas. Their figure of 110 mg. or  $30.2 \times 10^{-4}$  equivalents HCl per gram edestin is more than twice the present figure of  $13.4 \times 10^{-4}$ . The latter falls

<sup>6</sup> A recalculation by this second approximation method of the values given for gelatin (Ref. 1) did not change them significantly. The difference between the values for  $E'$  for 0.1 M HCl in Table I and in the previous paper is probably due to differences in the AgCl electrodes, which were prepared by the electrolytic method and used repeatedly without replating.

<sup>7</sup> Cohn, E. J., *Physiol. Rev.*, 1925, 5, 349.

<sup>8</sup> Hitchcock, D. I., *J. Gen. Physiol.*, 1922-23, 5, 383.

<sup>9</sup> Bancroft, W. D., and Barnett, C. E., *J. Phys. Chem.*, 1930, 34, 449.

on the horizontal part of their curve of pressure of HCl gas against weight of HCl taken up. If the present figure, which appears to result from the saturation with  $H^+$  of certain groups of the protein in solution, had any significance in their experiments, it might be expected to show up as a step in their curve, such as they found with gliadin and HCl. The absence of any such break in their curve seems to indicate that the protein behaves quite differently in solution and in the dry state. Both figures cannot represent the maximum combining capacity of edestin for HCl, and it is quite possible that neither does. Further experiments would be required to determine whether the protein is more altered by solution in 0.1 M hydrochloric acid or by saturation with dry hydrogen chloride gas. The fact that in the present experiments the E.M.F. remained constant for some hours would seem to indicate that, if there is any alteration in the protein, it takes place quickly and the system reaches a steady state. The discoloration of the dry protein which they report may be indicative of some secondary chemical reaction between the protein and the gas, which may possibly open up other acid-combining groups not sharing in the reaction in solution.

#### SUMMARY

Electromotive force measurements of cells without liquid junction, of the type  $Ag, AgCl, HCl + \text{protein}, H_2$ , lead to the conclusion that 1 gm. of edestin (or, more probably, edestan) combines with a maximum of  $13.4 \times 10^{-4}$  equivalents of  $H^+$  and  $3.9 \times 10^{-4}$  equivalents of  $Cl^-$ , when the protein is dissolved in 0.1 M HCl.



# THE INTRINSIC RHYTHM OF THE TURTLE'S HEART STUDIED WITH A NEW TYPE OF CHRONOGRAPH, TOGETHER WITH THE EFFECTS OF SOME DRUGS AND HORMONES

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It is well known, since the time of Ringer, that an excised heart, if properly supplied with oxygen in a physiological salt solution, will beat for several days, in fact until the tissue begins to disintegrate. Snyder<sup>1</sup> in connection with his studies on the effect of temperature on the heart beat of the Pacific terrapin (*Chemmys marmorata*) counted rates at intervals over periods of 11 hours and observed considerable changes in rate under fairly constant conditions. At  $25^{\circ}\pm 1^{\circ}\text{C.}$  the heart of the terrapin did not beat longer than 6 hours, due probably to the fact that this species is adapted to cold water.

The question arises as to the constancy with which a heart can beat over a period of many hours or several days, if all external factors affecting rate are kept quite uniform. For this type of investigation some sort of *continuous automatic* recording mechanism is a necessity. The Loomis chronograph, a preliminary description of which has already been published,<sup>2</sup> is specially designed to record rates of periodically recurring processes.

Anything which will actuate a relay will run the chronograph whose mechanism is essentially as follows:

The time for ten pulsations to occur is recorded by the length of a line of ink drawn by a moving pen on a revolving sheet of paper. During the next four pulsa-

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<sup>1</sup> Snyder, C. D., *Univ. Calif. Pub. Physiol.*, 1905, 2, 125.

<sup>2</sup> Loomis, A. L., and Harvey, E. N., *Science*, 1929, 70, 559.

tions the pen is returned to its zero position and is then ready to record the average time for ten more pulsations. Each line therefore represents fourteen pulsations and tells the average time for ten pulsations to occur. The machine is driven by a synchronous motor running on the A.C. lighting circuit. By reducing gears and pulleys the paper is revolved at the proper speed and the pen, attached to a pulley string, is drawn across at a constant rate of ten units per second. The string pulley which draws the pen is actuated by an electro-magnetic clutch in connection with a fourteen toothed wheel and an escapement, also worked magnetically. Each pulsation to be recorded is made to open an electric circuit that magnetizes the electro-magnet moving the toothed wheel one tooth. While the wheel revolves ten teeth the magnet clutch moves the pen at constant speed across the paper, and during the next four teeth the magnetic clutch is out and a spring pulls the pen back to the first position. If ten pulsations occur in 10 seconds the unit lines on the paper (ten to a second) represent a 1 per cent difference in rate and the paper can be calibrated by recording pulses of current at a rate of one per second. By mere inspection of the paper record one can observe any change in rate and very quickly calculate the percentage constancy of the rhythmic process.

Later models of the chronograph have been modified by (1) introducing an easily changeable gear box for driving the chronograph at different speeds; (2) returning the pen to its 0 position by revolving pulley wheel instead of by a spring, thereby shortening the recovery to two beats instead of four; (3) replacing the toothed wheel and escapement with an improved type of ratchet mechanism that cannot miss a beat; (4) placing a small electro-magnet in connection with the moving pen so that it is lifted off the paper with each beat, recording not only the average time for ten beats, but the interval between beats as well; (5) introducing a device for recording the time between single pulses or series of pulses without missing the time interval involved in the return of the pen to its zero position.

A complete description of the mechanical details of the chronograph will be published elsewhere.

In its new form the chronograph will record rates of 300 per minute to 1 beat in 6 minutes with absolute fidelity, giving a record which shows at a glance the change of rate without counting each individual beat. Figs. 2 and 3 give an idea of the records that can be made. The swing of a pendulum, manual beating of time, pulsation of a tambour, the fall of drops from a tube, the flashing of neon lamps, or the speed of rotation of any device can all be recorded by the proper electrical contact or relay mechanism. The growth of plants can also be recorded. It is only necessary to arrange a contact device of such a nature that, when the growing tip of the plant makes contact with a surface, the surface is automatically lifted a definite distance. A certain time interval will elapse before the plant grows sufficiently to make another contact. By knowing distance and time interval, which the chronograph records, we can determine (and record) the rate of growth. A continuous record of changes in light intensity can also be obtained by a very simple accessory mechanism.

The heart offers the best known case of a biological rhythm whose rate change is of fundamental importance. Action currents of the human heart or of animals picked up by the cardiometer of Boas<sup>3</sup> or by any properly designed amplifying device make beautiful records. For recording the fundamental heart rhythm we wished to avoid cardioaccelerator and cardio-inhibitory effects, and the following procedure, illustrated in Fig. 1, was followed.

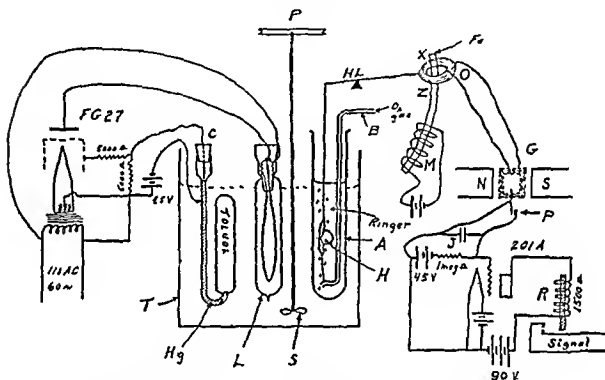


FIG. 1. Diagram of heart in thermostat showing wiring connections to chronograph. Explanation in text.

The heart of a turtle [either the painted (*Chrysemys picta*) or the slider (*Pseudemys rubriventris*) were used, and showed no characteristic differences] was removed, taking no special care to keep sinus intact, and mounted in a tube (A) of Ringer's solution<sup>4</sup> by tying threads to the tip of each auricle, one thread being wound around the end of an L-shaped glass tube (B) through which oxygen was bubbled into the Ringer, the other attached to the short arm of a heart lever (HL). It would seem that the easiest method of making an electrical contact would be through the long arm of the heart lever dipping into a pool of mercury. However, as is well known,

<sup>3</sup> Boas, E. P., *Arch. Int. Med.*, 1928, 41, 403; Boas, E. P., and Weiss, J. A. M. A., 1929, 92, 2162.

<sup>4</sup> The Ringer's solution contained NaCl, 0.65 per cent; KCl, 0.014 per cent; CaCl<sub>2</sub>, 0.014 per cent; NaHCO<sub>3</sub>, 0.02 per cent; and sometimes glucose, 0.2 per cent.

the turtle's auricle contains smooth muscle fibers whose periodic contraction causes tone changes in the auricles which are often *greater than the heart muscle contractions*, and are, of course, indicated by a change in position of the heart lever. This means that at times the lever arm will never leave the pool of mercury, and at other times the heart will never relax enough for the lever to touch the mercury surface, consequently spoiling the record and involving continual adjustment. To obviate this difficulty, a coil of fine wire (*O*) was attached to the end of the heart lever which moved the coil over the end of an electro-magnet (*M*), thus cutting the field of force and inducing a small current in *O*, sufficient to actuate a galvanometer relay (*G*), the Weston No. 30 model. By this device we always induce a small current in *O*, whether tone changes cause the coil to be moving in position *X* or in position *Z*. Recording has always been perfect after the adoption of this device.

The contact made by the galvanometer relay actuates another vacuum tube (201A) relay, shown in Fig. 1, which is built into the chronograph. The advantage of the vacuum tube relay, which controls the flow of plate current by charging the potential of the grid of a vacuum tube, lies in the fact that no current need flow across the contact points. Only a change of potential occurs and slight oxidation of these contact points does not introduce enough resistance<sup>5</sup> to affect the working of the relay (*R*), a high resistance (1500 $\Omega$ ) type. The magnet is actuated by plate current whenever a negative voltage of 45 volts is removed from the grid of the tube (201A), by short circuiting the 45 volts through a megohm resistance. If the galvanometer relay contacts are very short in duration the plate current can be made to flow long enough to actuate relay *R* by introducing a condenser (*J*) of 0.1 to 1 mf. capacity in the position shown in Fig. 1.

The same principle is adopted in the control of the thermostat (*T*) with the mercury-toluol regulator, the heating lamp (*L*) and stirrer *S*, with the pulley drive, *P*, except that a vacuum tube is used, a thyatron<sup>6</sup> (FG27) whose plate circuit is supplied by the 110 A.C. lighting mains with which the heating lamp is in series. The filament circuit is supplied by a 110 to 5 volt transformer and a negative grid voltage of 4½ volts is applied whenever the mercury rises in the regulator and makes contact with the contact wire, *C*. This cuts off the plate flow and puts out the heating lamp which will light again as soon as the contact is broken and 4½ volts negative removed from the grid. The outfit is an ideal regulating device for thermostats.

The temperature control of our hearts was 25°C.  $\pm 0.02^\circ\text{C}$ .; oxygen<sup>7</sup> was continually bubbled through at a constant rate; and the Ringer's solution was buffered so that constant pH was attained. As the heart was excised, cardio-motor reflex disturbances were eliminated. Everything worked automatically so that no

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<sup>5</sup> The contact can be made through the body of an individual.

<sup>6</sup> We are deeply indebted to the General Electric Company for this thyatron.

<sup>7</sup> In some experiments air was used instead of oxygen, but no difference in the rhythmic behavior of the heart was noted.

mechanical disturbances accompanying adjustment could have had an influence on the rate recorded, which we believe represents the fundamental intrinsic rhythm of some periodic process within the heart muscle cells. Twenty-five hearts in all have been studied at 25°C., some of them recorded for as long as 36 hours.

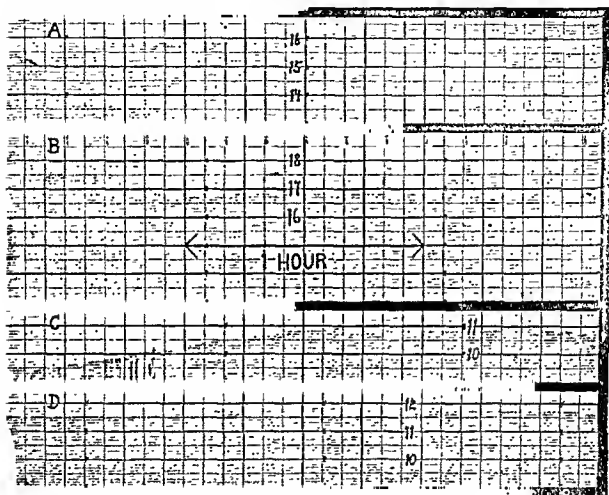


FIG. 2. Portions (2½ hours) of records of rate of turtle's heart in Ringer's solution without glucose under constant conditions at  $25^{\circ} \pm 0.02^{\circ}\text{C}$ . A. Sept. 23, 1929, 4 hours after start, showing considerable rhythmic changes in rate. B. Same heart after 8 hours, showing marked rhythmic decreases in rate exactly 20 minutes apart. C. Sept. 22, 1929, 40 minutes after start, showing rhythmic increases in rate exactly 50 minutes apart. D. Same heart, 4 hours later, showing remarkably constant rate. Ordinates, seconds for ten beats. Abscissae, 15 minute periods. Each vertical line represents fourteen beats of the heart and records the average time for ten beats to occur. The longer the ordinate line the slower the heart.

There are several important observations which have come from these records. (1) A considerable variation in the behavior of hearts from different turtles (Fig. 4). The rate is by no means constant over



a long time period. Neither does the rate continually decrease, although the general trend is in that direction. Usually the rate decreases during the first hour or two, but the rate may be the same at the

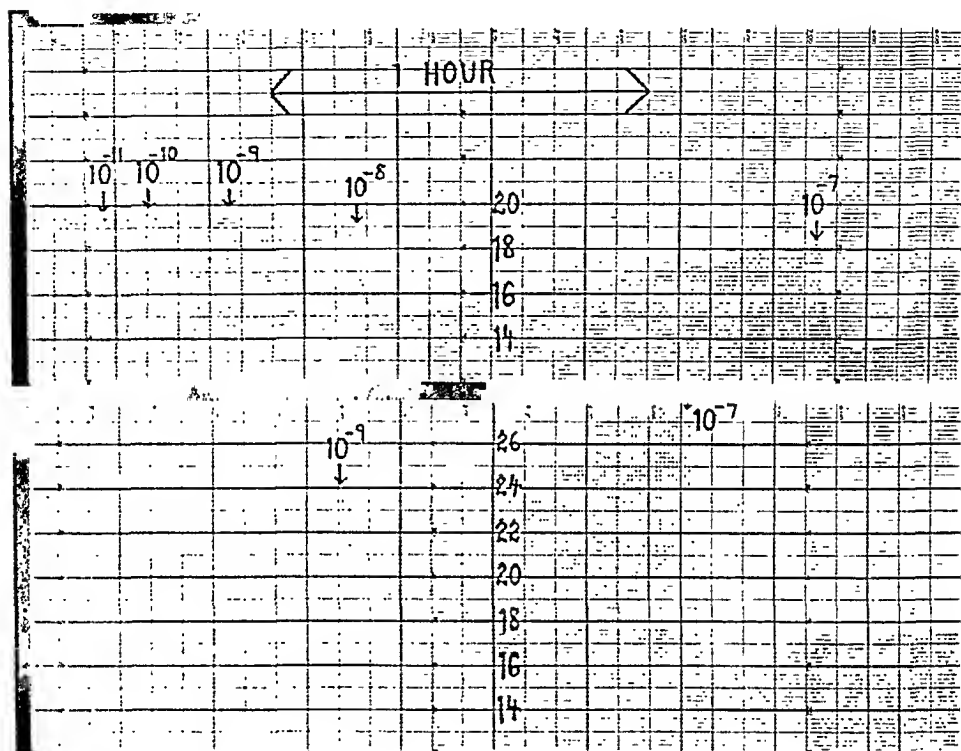


FIG. 3. Portions of a 32 hour record of the rate of a turtle's heart in Ringer's solution + 0.2 per cent glucose under constant conditions at  $25^{\circ} \pm 0.02^{\circ}\text{C}$ . Above—Fairly constant rate with slight rhythmic changes 3 hours after start, showing effect of adding increasing concentrations of adrenalin. Note first effect in increasing rate occurs with  $10^{-9}$  gm. adrenalin per cubic centimeter and marked effect with  $10^{-7}$  adrenalin. Below—Same heart with adrenalin effect gradually wearing off 90 minutes after end of A portion. Note again detectable increase in rate from adrenalin in  $10^{-9}$  gm. per cubic centimeter and very marked effect of  $10^{-7}$  adrenalin on a slowly beating heart. Abscissae, 15 minute periods. Ordinates, seconds for ten beats. The longer the ordinate line the slower the heart.

end of 12 hours, having made some fluctuations in the meantime. This initial decrease in rate may be connected with the seeping of adrenalin out of the tissue or the oxidation of adrenalin in the tissue. The ampli-

tude of contraction always decreases and is very weak when the heart is about ready to stop. Often beats will be omitted under these conditions, that is, there will be two beats and one omitted, or three beats and one omitted, etc. The greatest variation in rate noted was about 33 per cent in a 5 hour period and 60 per cent in a 12 hour period, and

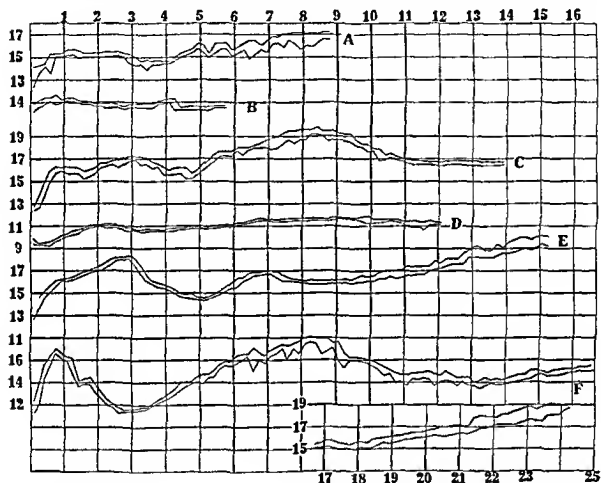


FIG. 4. Plots of rates of six hearts (A to F) Ordinates, seconds for ten beats. Abscissae, hours after heart removed and mounted in Ringer's solution at  $25^{\circ} \pm 0.02^{\circ}\text{C}$ . All conditions constant, no drugs or hormones added. A. June 16, 1929. B. June 14, 1929. C. June 17, 1929. D. Sept. 22, 1929. E. Oct. 7, 1929. F. Oct. 6, 1929. Abscissae for latter part of this record at bottom of chart. The curves were drawn by plotting the slowest rate (upper line) and the most rapid rate (lower line) for each 10 minute period.

the least variation was 2 per cent in a 5 hour period and 1 per cent over half hour periods (Fig. 2, D). This is quite remarkable regularity as it means that a heart will beat 100 times with a change of only one beat.

(2) The occurrence of rhythms in rate of beat which often recur at

rather definite intervals, that is periodic changes in the rate. A heart may show a 6 per cent decrease in rate lasting 3 minutes every 20 minutes for four 20 minute periods (Fig. 2, *B*), or similar 3 to 4 per cent increases in rate lasting a few minutes for three periods 50 minutes apart (Fig. 2, *C*). Such rhythmic changes in rate are not connected with the tone changes often observed in the auricles, whose

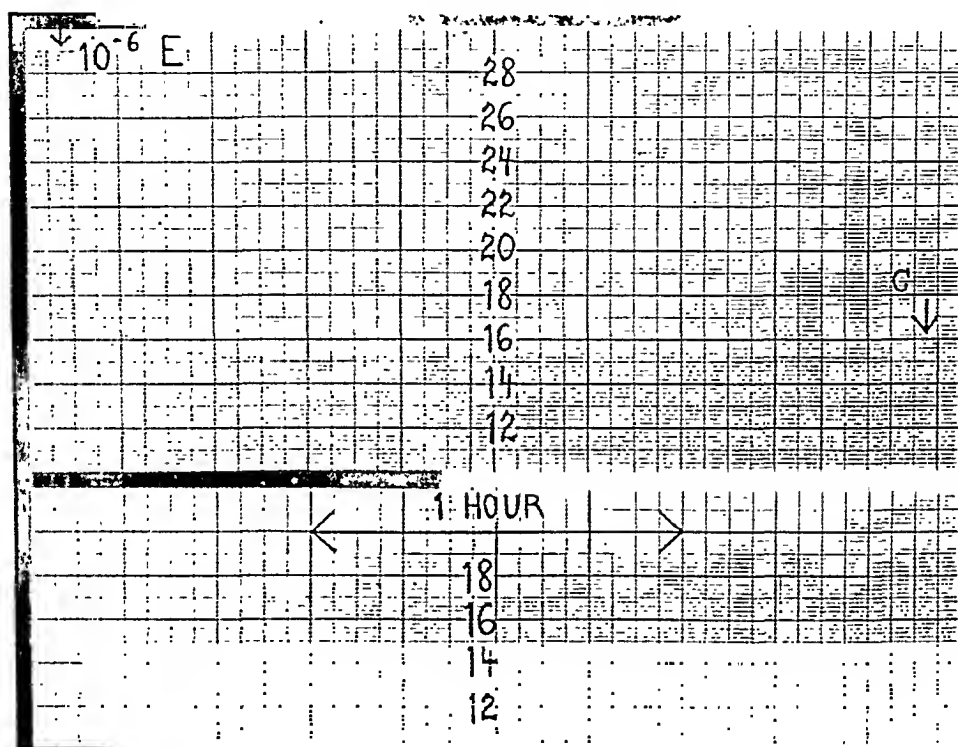


FIG. 5. Portions (2½ hours) of records of rate of turtle's heart showing: Above—effect of ephedrin ( $1:10^5$ ) at *E* and effect of chloretone ( $1:20000$ ) at *C*. Note remarkable regularity after ephedrin. Below—normal heart showing marked rhythmic changes in rate. Ordinates, seconds for ten beats. Abscissae, 15 minute periods. The longer the ordinate line the slower the heart.

period is 40 to 50 seconds, nor can they be traced to any change in the environment. They are true variations of the fundamental rhythm. Most frequently the maximum of these rhythms occur at intervals of 5, 6, or 10 minutes, but the period of such superposed rhythms is continually changing (Fig. 5).

These rhythmic changes are shown by practically all the hearts. Thinking that they might be connected with spontaneous activity of the postganglionic inhibitory nerve cells in the heart we tried adding atropin in concentrations of  $1:10^6$ ,  $1:10^5$ , and  $1:10^4$  to the Ringer's solution. These concentrations should paralyze the inhibitory nerve endings, but our records show no effect on the rhythmic changes in rate. The high concentration caused a progressive slowing of the rate.

Adrenalin or ephedrin added to the Ringer's does abolish the rhythmic rate changes, at the same time causing the well known increase in rate. One part of adrenalin in  $10^9$  parts of Ringer's causes a perceptible increase in rate (about 8 per cent) and  $1:10^8$  adrenalin causes a 21 per cent increase followed by a very regular and progressive slowing presumably due to slow oxidation of the adrenalin (Fig. 3). During this progressive slowing the heart beats with remarkable regularity, that is, no indication of periodic changes in rate. The percentage increase in rate after adding adrenalin is greater if the heart is beating slowly when the adrenalin is added than it is when the heart is beating more rapidly. These effects are illustrated in Fig. 3. To keep a heart in good rhythmic condition over long periods of time our observations suggest that a slight concentration of adrenalin should be present in the medium bathing the hearts.

As is well known ephedrin has a physiological effect which is similar to adrenalin. Our records show that  $1:10^8$  ephedrin added to Ringer's solution may double the rate of a slowly beating heart (10 beats in 30 seconds). The increase in rate takes 25 minutes for completion and the effect lasts for hours. Fig. 5 shows an ephedrin effect on a slowly beating heart. Note the difference in the form of the effect as compared with adrenalin. For greatest constancy in rate, a little ephedrin should be added to the Ringer's solution.

(3) Another characteristic of some hearts is a *sudden* change of rate. Both sudden increases in rate (15 per cent) and sudden decreases in rate (13 per cent) have been observed. They presumably indicate a sudden dropping out of one pace-making region or the sudden appearance of another. We cannot connect them with any changes in the environment.

$\alpha$  and  $\beta$  pituitary hormone<sup>8</sup> had no effect on the rate when freed of the chloretone which is added to preserve these solutions. The chloretone slows the rate slightly as shown in Fig. 5. The  $\beta$  hormone (pitressin) contained 10 pressor units per cubic centimeter and the  $\alpha$  hormone (pitocin) contained 10 oxytocic units per cubic centimeter. 2 cc. of each one was added to 200 cc. Ringer's solution in testing. Insulin (Eli Lilly Company), 20 units per cubic centimeter also had no effect on the rate when 2 cc. were added to 200 cc. Ringer's solution.

Our results with thyroxin<sup>8</sup> were somewhat variable. In six experiments using  $1:10^5$  thyroxin dissolved in a minimal amount of alkali, the increase in rate ranged from 0 to 26 per cent. The increased rate appears fairly quickly, but not as rapidly as with adrenalin, and is prolonged. It is possible that increased alkalinity of the Ringer's had some influence on the heart, as this factor was not sufficiently controlled.

Nicotin ( $1:10^4$ ) caused a progressive slowing, as did ergotamine tartrate ( $1:10^5$ ). Adrenalin administered after ergotamine showed the well known slowing of rate instead of an increase. Our records show that this chronograph is admirably adapted for studying the effects of drugs.

In conclusion we wish to express our thanks to Mr. Charles Butt, Technical Assistant in Physiology, Princeton University, for help in taking many of the records.

#### SUMMARY

A chronograph is described for recording continuously the rates of many different kinds of rhythmic processes over long time periods. The rate is read directly from the length of a line of ink, drawn by a moving pen.

Rates of beat of excised turtle's hearts in Ringer's solution have been recorded at 25°C. under constant conditions of temperature, pH,

<sup>8</sup> We are deeply indebted to Parke Davis and Company for a supply of  $\alpha$  and  $\beta$  pituitary hormones, and to E. R. Squibb and Sons for 100 mg. of crystalline thyroxine.

and oxygen supply for periods of 36 hours. Regular periodic variations in the fundamental rhythm are figured, as well as rates of extraordinary constancy. The effects of adrenalin, ephedrin, thyroxin,  $\alpha$  and  $\beta$  pituitary hormone insulin, nicotin, and atropin are described in the text.



## THE ACCUMULATION OF ELECTROLYTES

### I. THE ENTRANCE OF AMMONIA INTO *VALONIA MACROPHYSA*

By WM. C. COOPER, JR., AND W. J. V. OSTERHOUT

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Experiments on the penetration of  $\text{NH}_3$  into *Valonia macrophysa*, carried out in Bermuda in 1924-27, are described in the present paper. The interpretation of these results will receive further discussion in subsequent articles.

In order to ensure uniformity of material and of sea water a large number of cells, collected at the same time, were kept under the same conditions and all the sea water used for these cells and for the experiments was collected at the start and kept in carboys. The average volume of the cells was about 0.5 cc.

The experiments were carried out in stoppered bottles each containing 2400 cc. of solution with about 50 cells in each bottle. When the bottles were placed in the ocean they were fastened beneath a specially constructed raft so that the light received was mostly reflected from the whitish coral sand of the bottom. In the experiments in the laboratory direct sunlight was avoided. Growth and absorption of ammonia were more rapid in the ocean.

The temperature in the ocean varied from  $20^\circ$  to  $25^\circ\text{C}$ ., but in the course of any one experiment the variation was not more than  $2^\circ$  or  $3^\circ\text{C}$ ., except in those lasting several weeks. In experiments carried on in the laboratory the variation was much greater.

The  $\text{NH}_4\text{Cl}$  was added to the sea water in the form of a solution, 0.6 molar or normal in  $\text{NH}_4\text{Cl}$ ; the pH value of the sea water was adjusted by the addition of  $\text{HCl}$  or  $\text{NaOH}$ . After exposure to the sea water containing  $\text{NH}_4\text{Cl}$ , the sap was collected by means of a pipette having a sharp point which was thrust into the cell (in order to avoid loss of  $\text{NH}_3$  by suction, the sap was forced into the pipette with the fingers). Care was taken in collecting the sap to use some large and some small cells so that the sap<sup>1</sup> could be considered as taken from an average cell of about 0.5 cc. When sufficient sap had been extracted, it was blown out into a small beaker and covered. Two 5-cc. samples (for zero point determinations there were three) were pipetted out for sodium-potassium determinations, two 1-cc. samples for

<sup>1</sup> For analysis of the sap see Osterhout, W. J. V., *J. Gen. Physiol.*, 1922-23, 5, 225.



When  $\text{NH}_4\text{Cl}$  is added to the sea water it soon appears inside the cell,<sup>2</sup> where its concentration may become over 40 times as great as the sea water, thereby lessening the specific gravity of the sap<sup>3</sup> so much that the cells, leaving their normal situation on the bottom, rise to the surface, where they continue to grow. The change in the specific gravity of the sap is shown in Fig. 1.<sup>4</sup>

Experiments were made to determine the rate of penetration at

<sup>2</sup> For experiments on the penetration of  $\text{NH}_3$  into *Valonia* see Brooks, M. M., *Pub. Health Reports*, Washington, D. C., 1923, 38, 2074; for experiments on other cells see Warburg, O., *Z. physiol. Chem.*, 1920, 66, 305; Harvey, E. N., Carnegie Institution Publication, 1914, No. 183, p. 131; Irwin, M., *J. Gen. Physiol.*, 1925-26, 9, 235; Jacobs, M. H., Harvey Lectures, 1926-27, p. 146.

<sup>3</sup> Cf. Cooper, W. C., Jr., Dorcas, M. J., and Osterhout, W. J. V., *J. Gen. Physiol.*, 1928-29, 12, 427. Osterhout, W. J. V., Some fundamental problems of cellular physiology, New Haven, 1927. This curve is based on experiments in the laboratory.

<sup>4</sup> The change is more rapid when the cells, enclosed in bottles, are placed in the ocean; this may be due in part to light (cf. Hoagland, D. R., and Davis, A. R., *J. Gen. Physiol.*, 1923-24, 6, 47; Hoagland, D. R., Hibbard, P. L., and Davis, A. R., *J. Gen. Physiol.*, 1926-27, 10, 121.

various pH values. Since the initial rate is most easily interpreted the experiments were limited to 10 minutes which is as short a period as is practicable.

In each bottle containing 120 cc. of solution were placed 6 cells (each cell containing about 0.2 cc. of sap). Cells were selected as far as possible of the same size and shape. The bottles were placed on a slowly revolving wheel to keep the contents stirred. Ordinarily this treatment produced no injury which could be

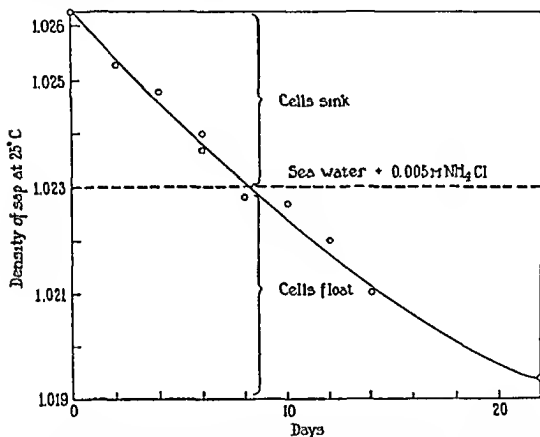


FIG. 1. Time curves showing the decrease in the specific gravity of the sap during the penetration of ammonia from sea water (at pH 8.3) containing 0.005  $M$   $NH_4Cl$ .

detected during the experiment or by subsequent observation (during several days) of cells kept in sea water. The ammonia (*i.e.*,  $NH_3 + NH_4OH + NH_4^+$ ) of the sap of untreated cells (about 0.0006  $M$ ) was subtracted from the ammonia values of the sap of treated cells to give the values shown in Fig. 2. The pH value of the sea water was adjusted by adding NaOH or HCl; the total ammonia of the sea water was kept constant.

The results are shown in Fig. 2 and it is evident that as the concentration of the undissociated base (*i.e.*,  $NH_3 + NH_4OH$ ) increases the

rate<sup>5</sup> of penetration of ammonia (total) also increases. But it is not easy to determine just how closely they agree. If we knew the rate of penetration when the total ammonia (*i.e.*,  $\text{NH}_3 + \text{NH}_4\text{OH} + \text{NH}_4^+$ ) is present as the undissociated base ( $\text{NH}_3 + \text{NH}_4\text{OH}$ ) it would be easy to see whether the rate is reduced to one half when the concentration of

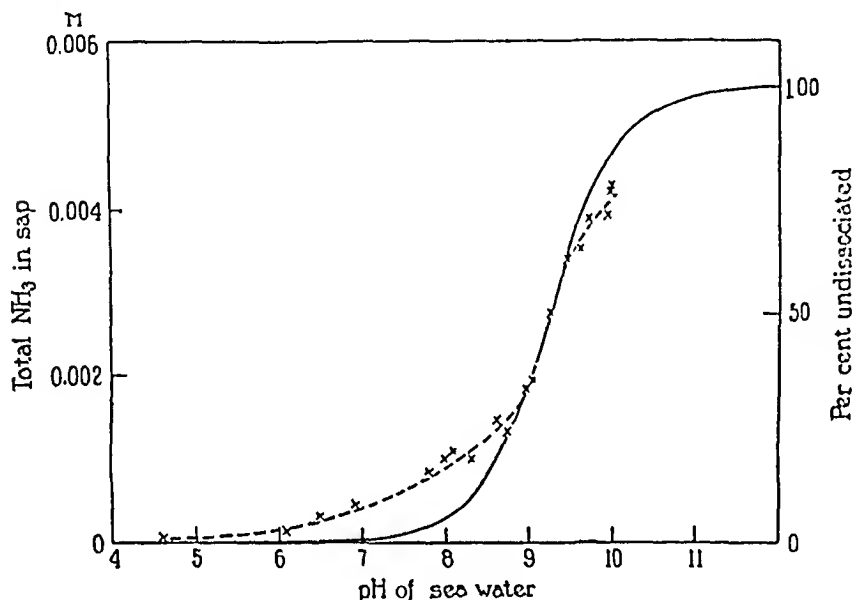


FIG. 2. Shows the increase in concentration of total ammonia in the sap after an exposure of 10 minutes (at  $21^\circ \pm 1^\circ\text{C.}$ ) to sea water containing 0.01 M  $\text{NH}_4\text{Cl}$  at various pH values. Broken line, observed values; continuous line, calculated curve for dissociation of  $\text{NH}_4\text{OH} + \text{NH}_3$  in distilled water  $\left( \frac{(\text{NH}_4^+)(\text{OH}^-)}{(\text{NH}_4\text{OH}) + (\text{NH}_3)} = K_B = 10^{-4.74} \right)$ . Since it happens that the inflection points of both curves lie at approximately pH 9.26 they have been made to coincide there: at that point the ordinate of the observed curve is 0.00275 M and this point is taken as 50 per cent of the calculated dissociation curve (whose inflection point and 50 per cent dissociation point coincide).

the undissociated base is reduced to one half, but unfortunately we cannot safely perform experiments at a sufficiently high pH value to ensure that practically all of the ammonia is present in undissociated form (even at pH 10.1, which is as high as we have gone, less

<sup>5</sup> Experiments with dead cells show little or no effect of pH value on penetration.

than 87 per cent of the total ammonia is regarded as present as the undissociated base).

It seems probable that in the absence of complicating factors the curve might resemble the equilibrium curves<sup>6</sup> for  $H_2S$  and  $CO_2$ , so that the inflection point would occur where the rate of entrance and the per cent of undissociated base was one half the maximum. We find that the point of inflection of the rate curve occurs at pH 9.25, which corresponds fairly well to the point (9.26) at which one half the total ammonia is in the form of the undissociated base ( $NH_3 + NH_4OH$ ) as calculated from conductivity data of solutions of ammonia in distilled water.<sup>7</sup> This would suggest that the initial rate of entrance reaches 50 per cent of its maximum when the undissociated base reaches 50 per cent of its maximum concentration.

In order to compare the two curves we have made them coincide at their inflection points as shown in Fig. 2.

We should expect the point of inflection of the dissociation curve to occur at a somewhat higher pH value in sea water on account of the greater ionic strength. In order to determine approximately this value for sea water  $NH_3$  was circulated in a closed glass vessel (designed by Dr. Grinnell Jones<sup>8</sup> to whom our thanks are due for allowing us to use his invention in advance of publication), containing on one side ammoniacal sea water and on the other, distilled water to which sufficient NaOH had been added to bring the pH value above 12 under which conditions all of the ammonia exists as the undissociated base ( $NH_3 + NH_4OH$ ). The total ammonia was kept constant and the pH value of the sea water was varied by adding HCl or NaOH; after equilibrium had been attained at any given pH value a sample was taken of the ammoniacal water in equilibrium with the ammoniacal sea water and the ammonia determined by colorimetric comparison. We therefore made measurements which gave a curve indicating that the inflection point, *i.e.*, the point where half the total ammonia is in the form of the undissociated base, is in the neighborhood of pH 9.5.

If the rate of entrance of ammonia is proportional to the external concentration of the undissociated base we might expect the curves to coincide, but this could be true only if the internal pH value remained constant. If, for example, the pH of the sap were kept constant at 12

<sup>6</sup> Cf. Osterhout, W. J. V., *J. Gen. Physiol.*, 1925-28, 8, 131; Osterhout, W. J. V., and Dorcas, M. J., *J. Gen. Physiol.*, 1925-26, 9, 255.

<sup>7</sup> This gives  $K_A = 10^{-14} - K_B = 9.26$  and  $K_B = 4.74$ .

<sup>8</sup> Jones, G., *J. Am. Chem. Soc.*, 1928, 50, 1600.

so that the entering ammonia did not dissociate inside, there would be no complications, but with sap at lower pH values a part of the entering  $\text{NH}_3$  would be transformed to ions and, therefore, the back pressure, or the tendency of the undissociated base to return to the sea water, would be diminished. In other words, the rate of penetration of ammonia would be a function not only of the external pH but of the internal pH as well.<sup>9</sup>

Our experiments show that the entrance of  $\text{NH}_3$  raises the internal pH value rapidly (Fig. 3), this effect being greater the higher the concentration of undissociated ammonia in the sea water. Hence we expect the rate to be relatively higher at the lower pH values of the sea water because under these conditions there is a smaller penetration of ammonia and consequently less effect on the pH of the sap (normally about 5.8). This would increase the ordinates of the curve progressively toward the left<sup>10</sup> and consequently make the slope of the curve less steep, *i.e.*, like that of the observed curve.<sup>11</sup>

The fact that the initial rate of penetration increases when the concentration of undissociated molecules increases suggests that it is chiefly undissociated molecules which penetrate, but it has been pointed out elsewhere that this does not necessarily follow.<sup>12</sup> It does indicate that the penetrating substance is  $\text{NH}_3$  or  $\text{NH}_4\text{OH}$  or the ion pair  $\text{NH}_4^+ + \text{OH}^-$ , rather than  $\text{NH}_4\text{Cl}$  whose rate of entrance would not be expected to increase as the external pH value increases.

That the substance penetrating at the start is largely  $\text{NH}_3$  (or

<sup>9</sup> Cf. Osterhout, W. J. V., *J. Gen. Physiol.*, 1929-30, 13, 261.

<sup>10</sup> We should expect a similar effect if penetration depended on chemical combination of undissociated molecules with some constituent of the protoplasm (or on absorption), since the amount of the compound formed would determine the rate of penetration and this would increase more and more slowly as the external concentration of undissociated molecules increased.

<sup>11</sup> This can easily be seen by making the ordinates of the dissociation curve progressively larger as we go toward the left and then multiplying all the ordinates by the same factor so as to make the maximum ordinate the same as for the dissociation curve.

<sup>12</sup> This results from the fact that if the ion pair  $\text{NH}_4^+ + \text{OH}^-$  enters, its penetration is proportional to the ionic activity product  $(^a\text{NH}_4) \times (^a\text{OH}) = K(^a\text{NH}_4\text{OH})$ , so that as the penetration of the indissociated base increases that of the ion pair will also increase. Cf. Osterhout, W. J. V., *J. Gen. Physiol.*, 1929-30, 13, 261.

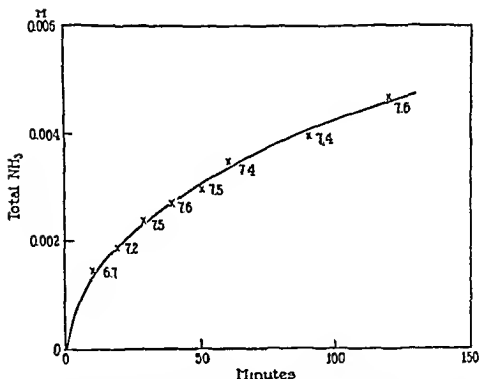


FIG. 3. Time curves showing the increase of pH value of sap during penetration of ammonia from sea water, at pH 8.1, containing 0.005 M  $\text{NH}_4\text{Cl}$ .

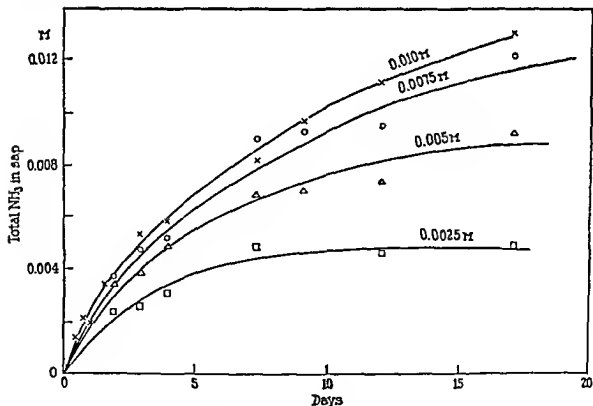


FIG. 4. Time curves showing increase of total ammonia during penetration from sea water (at pH 8.3) containing various amounts of  $\text{NH}_4\text{Cl}$ .

$\text{NH}_4\text{OH}$ ) rather than  $\text{NH}_4\text{Cl}$  is rendered quite certain by the data given in Fig. 3, showing the rise in pH value as the total  $\text{NH}_3$  in the sap increases (for it is evident that the penetration of  $\text{NH}_4\text{Cl}$  would not produce such an effect). The pH value of the sap soon reaches a maximum<sup>13</sup> and may subsequently fall off in a gradual manner.

As has been said already, the increase of undissociated ammonia does not raise the rate of penetration of ammonia as much as would be expected if there were no change in the internal pH value. The time

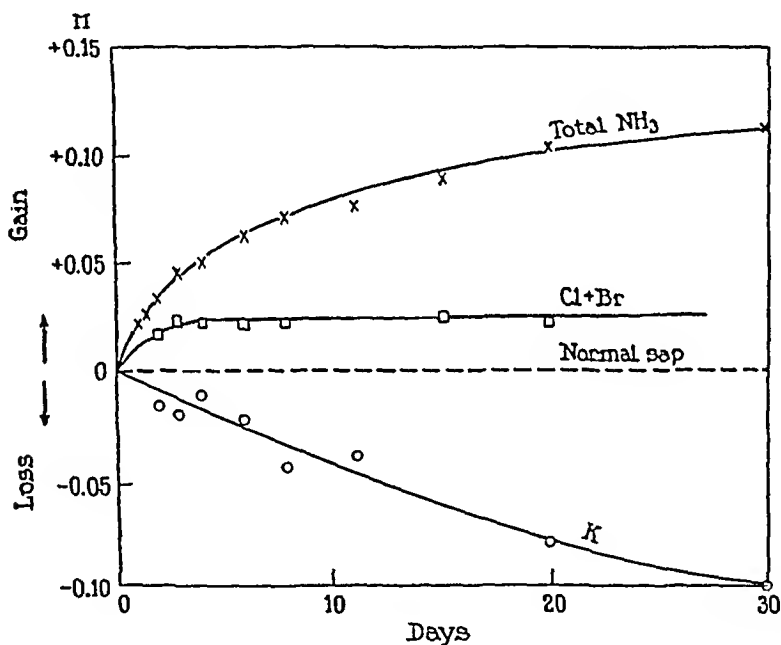


FIG. 5. Time curves showing increase in concentration of total ammonia and of halide, and decrease in the concentration of potassium during the penetration of ammonia from sea water (at pH 8.3) containing 0.005 M  $\text{NH}_4\text{Cl}$ .

curves in Fig. 4 bear out this statement. These curves do not reach a true equilibrium, for the cell keeps on growing; furthermore, when the concentration of  $\text{NH}_3$  in the sap reaches a certain point (less than 0.3 M) injury causes it to come out.<sup>14</sup>

<sup>13</sup> With 0.001 M  $\text{NH}_4\text{Cl}$  in the sea water this is 6.6, with 0.01 M  $\text{NH}_4\text{Cl}$  it is 8.2, the pH value of the sea water being 8.0.

<sup>14</sup> Cf. Irwin, M., *J. Gen. Physiol.*, 1925-26, 9, 235.

Fig. 5 shows that as the total ammonia in the sap increases, the concentration of potassium in the sap falls off, and measurements of volume<sup>15</sup> indicate that the decrease was not due merely to the absorption of water but that some potassium actually diffused out of the cell. The behavior of Na is not yet certain: further experimentation is in progress.

Fig. 5 also shows that the initial entrance of ammonia is accompanied by an increase in the concentration of halide, which soon reaches a fairly constant value dependent on the external pH. In the absence of ammonia in the sea water, there is no such variation in the halide of the sap with change in the external pH.

It may be added that the growth of the cells is increased by the addition of  $\text{NH}_4\text{Cl}$  to the sea water.

#### SUMMARY

When 0.005 M  $\text{NH}_4\text{Cl}$  is added to sea water containing cells of *Valonia macrophysa* ammonia soon appears in the sap and may reach a concentration inside over 40 times as great as outside. It appears to enter as undissociated  $\text{NH}_3$  (or  $\text{NH}_4\text{OH}$ ) and tends to reach a pseudoequilibrium in which the activity of undissociated  $\text{NH}_3$  (or  $\text{NH}_4\text{OH}$ ) is the same inside and outside.

When ammonia first enters, the pH value of the sap rapidly rises but it soon reaches a maximum and subsequently falls off. At the same time there is an increase of halide in the sap which, however, does not run a parallel course to the ammonia accumulation, but it comes to a new equilibrium value and remains constant.

The increase in  $\text{NH}_3$  in the sap is accompanied by a decrease in the concentration of K.

As  $\text{NH}_3$  enters the specific gravity of the sap decreases and the cells rise to the surface and continue to grow as floating organisms. The growth of the cells is increased.

<sup>15</sup> The cells grow faster when  $\text{NH}_3$  is added to the sea water.





# THE VARIATION OF ELECTRICAL RESISTANCE WITH APPLIED POTENTIAL

## II. THIN COLLODION FILMS

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### I

Analogies to certain physiological phenomena have been observed in the behavior of collodion membranes. Collander,<sup>1</sup> Michaelis,<sup>2</sup> and Northrop<sup>3</sup> have reported resemblances in the penetration of certain substances; Michaelis<sup>4</sup> has shown the similarity of the potential differences of such membranes to those of apple skin and other cuticle; Lahe and Zain<sup>5</sup> have imitated with collodion tubes such bioelectric effects as current of injury, polarizations in direct current, and the phenomena of electrotonus.

Furthering these analogies, the present paper amplifies some observations previously made<sup>6</sup> on very thin collodion membranes, which resemble the protoplasm of *Valonia* cells under some conditions<sup>7</sup> in

<sup>1</sup> Collander, R., *Soc. sci. Fennica, comment. biol.*, 1926, 2, 1; *Kolloidchem. Beih.*, 1924, 19, 72.

<sup>2</sup> Michaelis, L., and Weech, A. A., *J. Gen. Physiol.*, 1928-29, 12, 55. See also Fujita, A., *Biochem. Z.*, 1926, 170, 18.

<sup>3</sup> Northrop, J. H., *J. Gen. Physiol.*, 1927-28, 11, 233; 1928-29, 12, 435.

<sup>4</sup> Michaelis, L., *J. Gen. Physiol.*, 1925-28, 8, 33; *Bull. National Res. Council*, 1929, No. 69, 119.

<sup>5</sup> Lahe, R., and Zain, H., *Arch. exp. Path. u. Pharm.*, 1927, 125, 29, 53; 126, 284, 352.

<sup>6</sup> Blinks, L. R., *Proc. Soc. Exp. Biol. and Med.*, 1928-29, 26, 359. Shortly after the publication of this preliminary note, Ebbecke (Physiological Congress, Boston, 1929) described similar effects with collodion, stating that the current across a membrane from HCl to salt was greater than from salt to HCl.

<sup>7</sup> Blinks, L. R., *J. Gen. Physiol.*, 1929-30, 13, 793.

having an apparent resistance to direct current which varies with the voltage and the direction of an applied potential.

The conductance of collodion membranes was found by Michaelis<sup>8</sup> to vary greatly and characteristically with the solution of electrolyte in contact with them. His membranes, thoroughly imbibed by long washing, were usually in contact with the same solution on both sides. When they separated two different solutions (as in the experiments<sup>9</sup> where the transfer number of ions in the membrane was determined) there was at first a change of resistance with time, necessitating the adjustment of the current by a ballast resistance. This change was ascribed chiefly to polarization at the platinum electrodes.

In the present experiments, the most marked effects were found when the membranes separated two different solutions of different conductivity, such as HCl and KCl. The resistance then depended upon which ions were carried across the membrane, and the potential gradient under which they moved. It is believed that the changes are those of real ohmic resistance and that they present no phenomena requiring any special properties of collodion to explain, since the effects may be seen in other systems. The collodion film is thus used merely to obtain a very short conducting system separating two different solutions.

A rise of resistance always occurs when ions of lower mobility are moved into a conductor and supplant those of higher mobility. Devices for maintaining a constant current in a tube of electrolyte under these conditions are used in the determination of transport number by the "moving boundary" method.<sup>10</sup> A similar effect is more strikingly seen when a current passes through a fine capillary, filled with KCl solution and dipping into sea water; the resistance then rises when the positive current passes inward, and falls, when, it passes outward. This is an effect which must be controlled in the experiments performed with cells impaled on capillaries.<sup>11</sup>

<sup>8</sup> Michaelis, L., Green, A. A., and Weech, A. A., *J. Gen. Physiol.*, 1928-29, 12, 473.

<sup>9</sup> Michaelis, L., Weech, A. A., and Yamatori, A., *J. Gen. Physiol.*, 1926-27, 10, 685.

<sup>10</sup> Longsworth, L. G., and MacInnes, D. A., *J. Opt. Soc. Am. and Rev. Sci. Instr.*, 1929, 19, 50.

<sup>11</sup> Blinks, L. R., *J. Gen. Physiol.*, 1930-31, 14, 139.

In both the above cases the limits are represented by the resistance of the conductor when totally filled with one or the other solution. They tend to be reached at all current densities, the rise being slower with small voltages than with large. When sharp boundaries are maintained, this must be strictly true. But under very small potential gradients the boundaries become less sharp, and backward diffusion will prevent the complete substitution of one solution for another. (A heating effect may further contribute to this diffuse boundary at very high current densities.)

The most satisfactory explanation for the apparent equilibrium at any given potential in these collodion membranes is that a boundary of greater or less sharpness is formed, composed of different proportions of the ions involved. This maintains itself under the two opposed gradients of potential and diffusion, and as long as these remain constant, the character and position of the boundary is unchanged, giving a constant resistance. When either is changed the resistance changes. The process is exaggerated by the extreme thinness of the membrane, so that the boundary has but to move a short distance to the right or left in order to completely fill the membrane with one or the other electrolyte.

## II

The membranes were prepared from ordinary Merck's collodion U. S. P.; or from Parlodion dissolved in 75 per cent alcohol, 25 per cent ether. The kind of collodion seemed to be immaterial for these effects. A small amount of the solution was poured upon a clean glass plate and another plate gently placed over it (avoiding bubbles), spreading the collodion into a film. The plates were then carefully slid apart and stood on end to dry. 15 minutes' drying was often sufficient for these very thin films, but usually they were allowed a day or more. They adhere very tightly to the glass and may be stored in this condition. Usually, however, they were removed from the glass and stored spread out on filter paper. This was done by immersing the glass plates in water until the films were free (loosened, if necessary, by a camel's hair brush) and floated to the top. They were then spread into a smooth sheet, and lifted out, supported on filter paper inserted from below. Dried flat they now remained slightly attached to the paper, and could be stored in folders until needed.

From regions which showed brilliant red and green interference colors 2 cm. squares of paper and film were cut with shears and the film cemented over the ends of tubes for use. These tubes were usually of the Y-form shown in Fig. 1. One

arm of the Y was gently heated and the end ringed with the low melting point wax "Picein" which adheres well to both glass and collodion. While still warm it was brought gently in contact with the square of collodion which formed a firm seal to the glass and came free of the filter paper. The other arm of the Y was filled with a plug of stiff agar gel, imbibed with the solution used in the Y-tube. The Y-tube was usually filled for a distance of about 1 cm. up the vertical neck. For emptying it was inverted and drained. Great care was needed to avoid vibration at all times as the thin membranes were easily broken by jar, even with the small head of liquid used.

The arms of the Y-tube dipped into intermediate vessels filled with the desired solutions as shown. The electrodes were large ones of chloride-coated amalgamated lead, and were practically free of polarization at any current density used in these experiments. No resistance changes of great magnitude were observed in

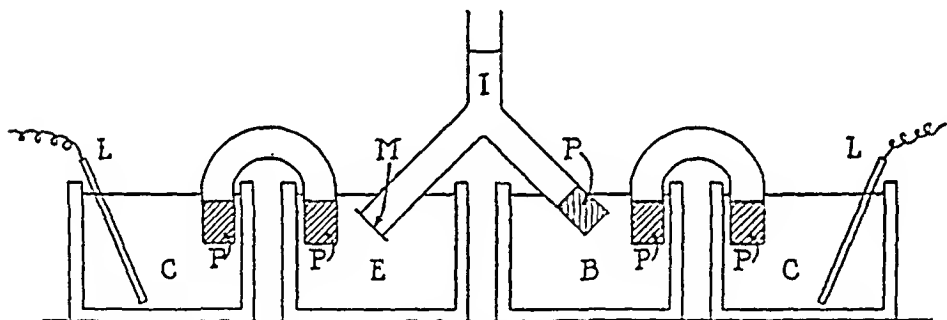


FIG. 1. Diagram of apparatus for measuring resistance of collodion films. The membrane (*M*) is cemented over one arm of the Y-tube, and separates the inner solution (*I*) from the outer solution (*E*). The other arm, plugged with agar (*P*), dips into the cup (*B*). U-tubes, plugged with agar (*P*), connect with the cups (*C*) into which dip the electrodes (*L*).

the system of conductors leading up to the membranes. With both arms of the Y-tube plugged with agar the resistance of the system under various conditions was between 500 and 1500 ohms; it remained constant to 50 or 100 ohms during long current flows under applied potentials of as much as 4 volts. This value formed the base which was subtracted from the total resistance to give that of the membrane. The resistance was measured in the direct current bridge previously described.<sup>12</sup>

The membranes introduced resistances from a few hundred ohms (when there were large leaks) to several megohms, as measured under standard conditions (in contact with 1.0 M KCl solutions). Neither of these extremes showed the phenomena of variable resistance as strikingly as the membranes of intermediate values most of which, as used, ranged between 3000 and 50,000 ohms, under standard

<sup>12</sup> Blinks, L. R., *J. Gen. Physiol.*, 1929-30, 13, 361.

conditions. With care the solutions in contact with the membrane could be changed 8 or 10 times, and a return made to the first resistance value when in contact with the initial solutions. Sometimes a jar or the passage of a large current itself suddenly reduced the value of resistance under all conditions to a new level, at which the effects still took place, although often with a changed percentage. The resistance of the membranes had therefore to be checked frequently under standard conditions (in contact with 1  $M$  KCl). When this was done a considerable series of comparisons could be instituted.

### III

When a small potential was applied to a membrane separating two solutions, a definite resistance was soon reached, at which the bridge maintained a constant balance. When the potential was changed, a new value was produced, either higher or lower, depending upon the direction and intensity of the potential gradient: 15 seconds was usually sufficient to reach this new constant level, and this time was chosen as a standard in which to make the balance. In some cases a longer time was required, but in general the values at 15 seconds represented fully 95 per cent of the steady values.

The largest absolute changes of resistance were usually found in freshly made membranes immediately after being sealed to the tubes. As an example, such a new membrane had a net resistance of 9500 ohms when separating a solution of normal HCl inside the tube from sea water outside. This was the value when 50 mv. was applied to it, in such a direction that the positive current passed outward (from HCl toward sea water), moving H ions across the membrane. When the potential was increased in this direction the resistance fell further, reaching at 100 mv. 7800 ohms, and at 0.5 volt a value of 5200 ohms. At higher voltages the resistance became nearly constant at about 4800 ohms. On the return to a lower applied potential the resistance rose again and at 100 mv. was 7700 ohms. The potential was now reversed so that the positive current passed from sea water inward to HCl. At 50 mv. the resistance was 15,000 ohms; at increased voltages it rose rapidly, reaching at 0.5 volt 18,300 ohms, and at 3.3 volts 21,500 ohms. On the return to 100 mv. the value was 15,600 ohms.

After this membrane had been placed in sea water for 2 days, the resistance rose only to about 15,500 ohms at the maximum inward potential. A day later, after many measurements had been made, the

range was from 500 ohms (current outward) to 5800 ohms (current inward). There had evidently been progressive injury which increased the absolute permeability to ions. But the *percentage* of resistance rise under the same conditions had increased, probably because the current densities in the earlier experiments had not been sufficient for sweeping out all H ions from the membrane.

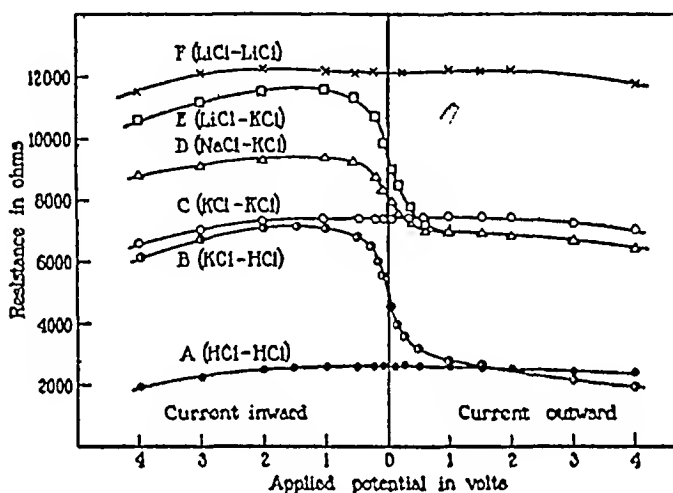


FIG. 2. Variation of resistance with applied potential in a membrane separating molar solutions, as follows:

| Curve  | Outside solution | Inside solution |
|--------|------------------|-----------------|
| A..... | HCl              | HCl             |
| B..... | KCl              | HCl             |
| C..... | KCl              | KCl             |
| D..... | NaCl             | KCl             |
| E..... | LiCl             | KCl             |
| F..... | LiCl             | LiCl            |

Potentials increasing to the left of zero cause a flow of positive current *inward*; to the right *outward*.

This experiment may be considered analogous to many biological cases, where a cell encloses sap of high electrolyte concentration and is surrounded by water of low electrolyte concentration.

Even greater rises of resistance may be produced by greater dilutions of the sea water outside the membrane. But more useful information was gained by keeping the concentrations equal on both sides of the

membrane, and varying the *composition* of the solutions. In most of the experiments the concentration was kept at 1.0 molar, and chlorides only were employed, the cations being varied. Such a group of experiments is shown in Fig. 2 for HCl, KCl, NaCl, and LiCl. It is seen that the resistance increases in the order given. When two salts were separated by the membrane the resistance rose when the direction of the current was such as to carry the less mobile ions across the membrane. In general the highest value then reached (at high potentials)

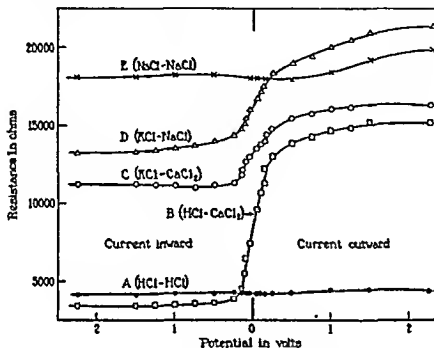


FIG. 3. Variation of resistance with applied potential in a membrane separating molar solutions of various kinds: Curve A, HCl both sides; B, HCl outside,  $\text{CaCl}_2$  inside; C, KCl outside,  $\text{CaCl}_2$  inside; D, KCl outside, NaCl inside; E, NaCl inside and outside. The membrane had evidently been somewhat injured between Curves A and B, and between D and E. Potentials increasing to left of zero cause inward current, to right, outward.

was a little lower than that found (at all potentials) when the membrane was exposed to the more poorly conducting solution on both sides. This showed that there was still some mixing of ions in the membrane at the highest potentials. The resistances of the membranes, when wholly imhihed with the given solutions, were about in the proportions of the specific resistances of aqueous solutions of the equivalent concentration. The membranes thus did not show any specific properties in exaggerating differences in ionic mobilities, such



as Michaelis found in his celloidin (Schering) membranes. The same kind of collodion, the higher salt concentrations used, and the thinness of the membranes may all account for the absence of this effect here. The membrane in 1 M LiCl had about twice the resistance than in 1 M KCl, and in the latter had about three times that in 1 M HCl. The value with NaCl fell between KCl and LiCl, while in 1 M  $\text{CaCl}_2$  (Fig. 3) it had a slightly lower resistance than in 1 M HCl. These are roughly proportional to the specific resistances of the solutions.

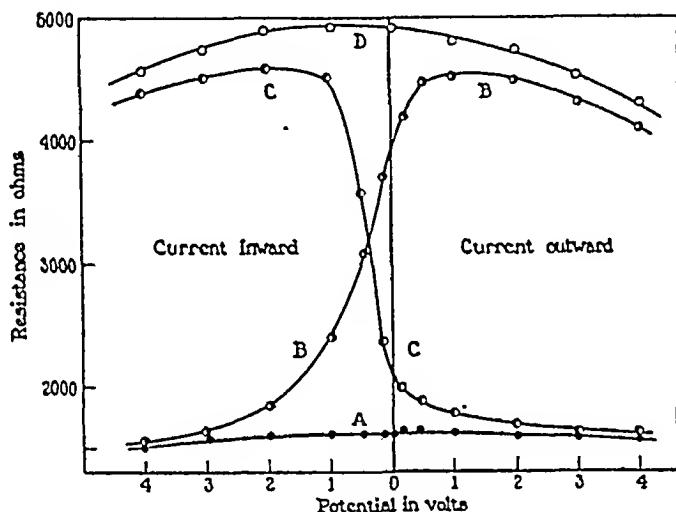


FIG. 4. Variation of resistance with applied potential in a membrane separating molar solutions of HCl and KCl. Curve A, HCl on both sides; B, HCl outside, KCl inside; C, KCl outside, HCl inside; D, KCl on both sides. Potentials increasing to left of zero cause inward current, to right, outward.

The alternating current resistance of these membranes was usually equal to that of direct current just where the curves crossed the zero potential.

While the resistances are in general dependent on the solutions with which the membranes are in contact, and hence are reversible when the solutions are changed side for side, there are also slight, and sometimes very large, asymmetries, which greatly distort the curves. Fig. 4 shows the values reached when KCl and HCl were applied to the membrane in four different combinations. With HCl inside the

and KCl outside the curve crosses the zero line at quite a different point from where it does with the opposite arrangement. The two curves also cross each other at a point about 300 mv. from the zero line.

It is also to be noted that the curve D is distinctly rounded, with the highest resistances at the lowest potentials. This was often found to be the case in the membranes, and may be due to a widening of cur-

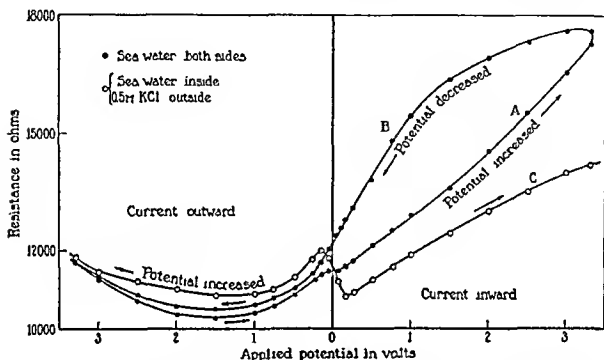


FIG. 5. Anomalies in the variation of resistance with potential. Curves A and B are for a membrane with sea water on both sides. Curve A: potentials decreased from left to zero, then reversed and increased from zero to right as shown by arrows. Curve B: potentials decreased from right to zero, while the current continued to flow. There is a hysteric effect, the resistance remaining higher than during the increase of potential. To left of zero, however, with increase of potential during current flow, Curve B agrees well with Curve A. Curve C was taken after 0.5 M KCl had been substituted for sea water outside the membrane. The curve goes through a double inflection near the zero line. Potentials to left of zero cause *outward* current, to right, *inward*.

rent paths under high potentials. But the opposite shape, with the resistance *rising* on both sides of zero was occasionally found, and is so far without explanation. Fig. 5 shows a case of this kind, and also illustrates two other anomalies. It is evident that the resistance rise is not symmetrical about zero potential, being mostly confined to an *inward* current, decreasing at first with *outward* currents, and finally

rising again at higher potentials. When 0.5 M KCl was substituted for sea water on the outside of the membrane the curve went through a double inflection at the lower potential values. The remainder of the curve paralleled the sea water curve at slightly different absolute values. This double inflection was characteristic of several membranes just after solutions had been changed and is undoubtedly due to the establishment of new diffusion gradients in the membrane.

Another anomaly, shown in Fig. 5, is the different shape of the curve on ascending and on descending potentials. In the descending arm (*B*) of the sea water curve, the current was allowed to flow continually while the potential was decreased, not being broken between readings. These were made in the standard time (15 seconds) after the new potential was established across the membranes. It is seen that the resistance stayed at a much higher value at most potentials, and then began to decrease more rapidly, at the approach to zero. On the reversal of potential the original values were nearly duplicated.

This delay, which resembles a hysteretic effect, is seen in another way when potentials are applied in rapid succession. While the same resistance level is eventually reached, this takes longer at the first application than at later ones following immediately after. Fig. 6 is a string galvanometer record of the bridge deflections, showing two applications of the same potential to a membrane separating solutions of different conductivity. In this case the less mobile ions were being carried into the membrane by the current and the resistance rose during the flow of current. But the time to rise approximately to balance point (at break) was nearly 14 seconds, while the same point was reached in about 7 seconds on a new application, following 1 second after the first was broken. It is evident that during the 1 second between potential applications much diffusion had occurred, which had reduced the resistance again, and unbalanced the bridge; but this had not gone as far as in the original condition, and it did not take as long to establish the new gradient in the membrane. Very similar hysteretic effects have been noted in *Valonia* cells,<sup>7</sup> and the curves have much resemblance.

Comparison of this record with those of living cells does, however, show a striking difference as respects the back E.M.F. of polarization. When present this appears on the string records as a large upward excursion of the image at the removal of an applied potential. In

*Nitella*<sup>13</sup> and in normal *Valonia* this upward curve at "break" is nearly symmetrical with the downward movement at "make;" in this it resembles the curves for electrode polarization. But the upward movement at break is nearly absent in the records for collodion, showing that the rise of resistance is one of "real" or ohmic resistance.

In this respect these collodion membranes are in agreement with those of Espermüller<sup>14</sup> which were held only between metal plates, without any contact with solutions. The ions were there necessarily

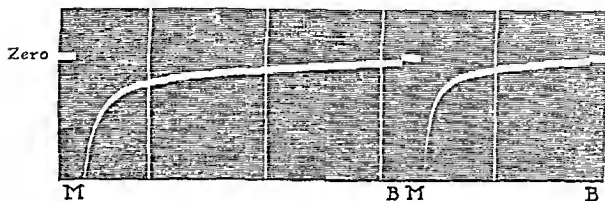


FIG. 6. String galvanometer record of bridge deflections during two applications of 0.5 volt to a membrane separating 1.0 M KCl from 1.0 M HCl. (Positive current from KCl to HCl.) The image deflects from zero (no current in bridge) downward at the application or "make" (M) of the potential and rises as the resistance increases nearly to the balance point. At removal of the potential or "break" (B) there is a negligible back E.M.F. displayed as the image returns to zero. At the second make (M) the deflection is less and the resistance rise is about twice as fast. At break (B), there is again a negligible back E.M.F. Time marks, 5 seconds.

derived from the collodion itself, and the resistance rose or fell as they were carried to one side or the other of the film. Polarization was absent.

#### IV

The biological significance of the phenomena described for collodion will depend upon the relative rôles played in living cells by the two components of any hindrance to the passage of direct current. These may be (1) a real ohmic resistance and (2) an E.M.F. of polarization.

<sup>13</sup> Blinks, L. R., *J. Gen. Physiol.*, 1929-30, 13, 495.

<sup>14</sup> Espermüller, E., *Arch. Elektrotech.*, 1928, 21, 148.

Both may occur in any conducting system and certainly the first is always present (with a possibility of variation). It would be possible to ascribe many of the observed resistance variations in living matter indirectly to variations in the bioelectric potential difference existing at the cell surface, which, by a small change of value (as at or near the zero point in these curves), might bring about a large change in the electrolyte content of that surface. This would affect not only direct current measurements, but also those of low frequency alternating current in which the ohmic resistance of the membrane is more important than its capacity. A tempting hypothesis could thus be established on the relations between bioelectric potential and electrical resistance.

The evidence, however, for *Valonia* and *Nitella*, as well as for many other organisms, indicates the greater importance of polarization E.M.F.'s in the observed "resistance" to direct current. The study of impaled *Valonia* cells<sup>11</sup> especially indicates the important rôle of these back E.M.F.'s. It is felt therefore that the mechanism of rise of resistance in the thin collodion films reported here does not completely represent that of the cells. It is, however, described since it may have a bearing upon other cases. It should further be noted that to a certain extent collodion furnishes a model for polarization potentials, inasmuch as Labes and Zain<sup>5</sup> found back E.M.F.'s of as high as 80 mv. developed by long passage of current through another type of membrane. Further study of these as a model of the *Valonia* cells is in progress.

#### SUMMARY

The resistance of very thin collodion membranes to direct current bears some resemblance to that of living cells since it varies with the applied potential. With membranes separating two different solutions the resistance varies with the direction (and the voltage) of the applied potential, rising when less mobile ions are carried across the membrane and falling when more mobile ones are so carried. (With some membranes the resistance varies with potential when the same solution is on both sides.) These changes are very prompt and regular. There is a hysteretic effect of previous current flow.

But the membranes differ from *Valonia* cells in that the rise of resistance is largely ohmic, there being little or no polarization potential.

# THE VARIATION OF ELECTRICAL RESISTANCE WITH APPLIED POTENTIAL

## III. IMPALED *VALONIA VENTRICOSA*

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### I

In many freshly gathered cells of *Valonia ventricosa* the apparent resistance of the protoplasm to direct current varies with the value of the potential applied to measure it.<sup>1</sup> It was further shown<sup>1</sup> that the apparent resistance of a single layer of protoplasm in such cells depends also upon the direction of the current, falling somewhat when the applied potential is increased to carry the positive current outward across it, but rising greatly during the first increase of 100 mv. in the opposite (inward) sense.

These directional effects were studied in cells in which the contact-region at one end had been killed with chloroform. They were therefore limited to the time during which the intact end remained uninfluenced by the diffusion of chloroform, or by the death of the other end. This was at most 15 minutes to half an hour. In order to extend greatly the time of these measurements across a single layer of protoplasm, and to avoid the influence of toxic agents, a series of resistance measurements was made with *V. ventricosa* cells impaled upon fine glass capillaries. Through these the current was led into (or out of) the vacuole, and passed but once across the protoplasm to (or from) the surrounding sea water.

It is recognized that the advantages of this method are balanced by the possible injury inflicted by the insertion of a capillary into the cell. It has already been noted<sup>2</sup> that cells which have reached the

<sup>1</sup> Blinks, L. R., *J. Gen. Physiol.*, 1929-30, 13, 793.

<sup>2</sup> Blinks, L. R., *J. Gen. Physiol.*, 1929-30, 13, 361.

"constant" state may be rendered again "variable" (*i.e.*, having an apparent resistance depending on the value of the applied potential), simply by impalement. However, the production of this state at will has contributed toward an understanding of its causes, and it is believed that an objective statement of the changes occurring as the cells remain impaled may be valuable. The formation of an "electrical seal" about the capillary has already been referred to in the static measurements of Osterhout, Damon, and Jacques.<sup>3,4</sup> The present measurements of resistance and polarization during the flow of current across the whole protoplasmic film yield further criteria on the condition of impaled cells.

## II

The present technique was directly adapted from that of Osterhout, Damon, and Jacques, the impalement being done under a slight pressure and a small hydrostatic head subsequently maintained to prevent the collapse of the cells. Various arrangements utilized are shown in Figs. 1, *a*, *b*, and *c*. Supported by the capillary above, and by a ring of cork or glass below, the cell was held gently and motion upon the capillary was prevented. This immobility contributed largely to the prompt recovery and long life of the cells. The use of rather small cells (from 0.5 to 1 cm in diameter) was also helpful, since the protoplasmic film of larger cells seems to be much more unstable, and breaks up into hundreds of tiny cells (inside the old cell wall) very promptly after mechanical injury such as pricking. Smaller cells (2 to 3 mm. in diameter) could sometimes be impaled without support from below (Fig. 1 *d*).

The capillaries usually projected 2 or 3 mm. into the vacuole; they were about 1 cm. long and had an outside diameter of 0.2 to 0.5 mm. They were drawn on the ends of  $\frac{1}{4}$  inch (or smaller) glass tubing, which, filled with artificial sap of *Valonia ventricosa*,<sup>5</sup> formed the salt-bridge connecting the vacuole to one of the electrodes.

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<sup>3</sup> Osterhout, W. J. V., Damon, E. B., and Jacques, A. G., *J. Gen. Physiol.*, 1927-28, 11, 193.

<sup>4</sup> Damon, E. B., *J. Gen. Physiol.*, 1929-30, 13, 207.

<sup>5</sup> This had the composition: 0.6 M. KCl, 95 parts, to 0.6 M. NaCl, 5 parts. For the original analysis of this sap see Cooper, W. C., Jr., and Blinks, L. R., *Science*, 1928, 68, 164.

In this they resemble most of the so-called "micro-electrodes," which are generally microscopic only as respects the capillary point inserted into the cell, and are more properly "micro-salt-bridges" connecting the cell interior to more or less distant electrodes of quite ordinary size.<sup>6</sup> This is indeed a more satisfactory arrangement than a minute metal surface, in any electrical measurement where polarization is to be avoided; in the present studies particularly, where current flow is purposely caused in order to produce the described effects, such polarization

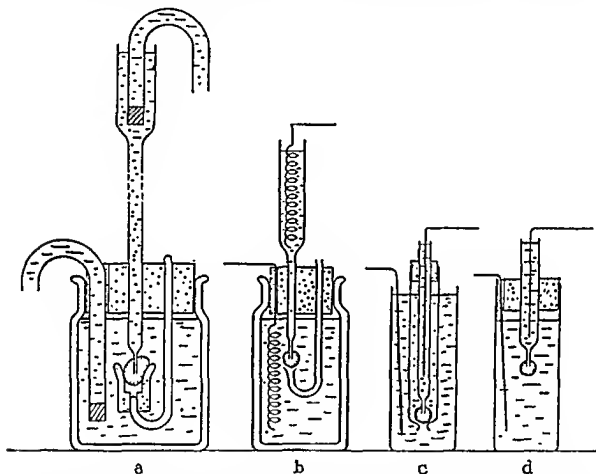


FIG. 1. Apparatus used for supporting the impaled cells of *Valonia*. In each case the capillary is drawn on the end of a vertical tube which fits rather tightly in the cork stopper. It is filled with artificial sap. In *a* the cell is supported from below on a cork with flexible prongs, itself supported on a glass rod inserted firmly into the cork stopper. In *b* the support is the flared end of a glass tube similarly inserted. In *c* the cell is supported inside the narrow orifice of a wider glass tube. In *d* there is no lower support for the small cell. Except in *a* the electrodes are of straight or coiled silver wire, inserted into the sea water or artificial sap as shown. In *a* there are agar-plugged salt bridges connecting to lead electrodes (not shown).

<sup>6</sup> But the silver plated quartz needle recently described by Sen is really a micro-electrode. Sen, B., *Proc. Soc. Exp. Biol. and Med.*, 1929-30, 27, 310.



is inadmissible. It was found that very small electrodes of fine silver wire coated and introduced through the capillary into the vacuole of impa gave very erratic results and seriously distorted the observed effects. There is also the danger of injury to the protoplasm by minute amounts of silver salts dissolving from metal electrodes inserted directly into the vacuole periods of time, and the impossibility of cleaning and recoating the surface at frequent intervals while in the cell. Instead, more distant electrodes of larger surface were employed and the desired currents obtained through capillaries by the application of higher voltages.

The electrodes were usually of straight or coiled silver wire (20-gauge), 1/2 inches in length, and inserted respectively into the sea water and into the sap in the tube above the capillary (Figs. 1 *b, c, d*). They were cleaned and coated with chloride electrolytically at least once a day (or oftener when polarization, measured with a blank capillary, warranted). In some of the even larger electrodes of sheet lead, amalgamated and chloride-coated were employed; these dipped into separate vessels and were connected to the agar-plugged salt-bridges, shown in Fig. 1 *a*.

At either type of electrode, polarization was virtually absent during flows of the largest currents used in the measurements (50 to 100 microamperes). Similar constancy of ohmic resistance was observed during such flows of current through the capillaries, either when these were in connection with artificial vessels, or with the natural sap of the vacuole. Thus in a cell killed immediately before measurement, the current was passed through a capillary inserted into the vacuole, for as long as 15 minutes, and in either direction, without significant change of resistance. Such constancy is of course essential for determination of amount of change in the living protoplasm. Very satisfactory agreement of basal value from day to day was likewise found, the only fluctuations being those expected from 2° or 3° changes of temperature.

The constancy of this resistance disappeared when the sap-filled capillaries were dipped directly into sea water, or remained in a dead cell long enough for sea water to diffuse into the vacuole. In these cases the resistance rose when positive current was passed inward, and reached a value about 20 per cent higher when most of the  $K^+$  ions had been supplanted in the capillary by the sodium ions of the sea water. Such a diffusion of sea water into the sap is a sign of injury, and this effect was never found in healthy cells. Since the rise of resistance in the capillary is entirely ohmic, it is easily distinguished (by the absence of E.M.F.) from the protoplasmic phenomena, which will later be shown to be reactive (i.e., due to the production of a back E.M.F.).

Further check on the basal resistance of the capillary was given by alternating current measurements at a frequency (5000 cycles) where the impedance of the protoplasm was negligible. These gave very constant readings from day to day independent of the changes of apparent resistance to direct current occurring in the protoplasm.

Depending upon the diameter and length of the capillary, this basal resistance ( $S$ ) ranged from 10,000 to 100,000 ohms. In order to pass sufficient current through such capillaries to obtain the described effects in the protoplasm it was sometimes necessary to apply as much as 4 or 5 volts to the electrodes. The potential drop across the protoplasm was, of course, but a small fraction of this. The total applied potential ( $V$ ), and the apparent direct current resistance ( $R$ ), were determined in the Wheatstone bridge previously described.<sup>2</sup> This bridge also provided for the measurement of potential differences existing in the cell, either by compensating to zero with a series potentiometer, or by deflection of the direct reading detector (vacuum-tube electrometer with string galvanometer). This could be done at full sensitivity, with one arm of the bridge thrown out (open circuit), or, in the condition of balance, *with sensitivity reduced one-half*.

The values of net protoplasmic potential difference were obtained by subtracting or balancing out the P.D. between the electrodes, due to the different chloride content of sea water and artificial sap. This P.D. was usually about 4 mv.

For purposes of comparison with previous papers,<sup>1,2</sup> the results with *Valonia ventricosa* are here described. The cells were used in June, July, and August at Dry Tortugas, Florida, at temperatures between 25° and 30°C., and later in New York at lower temperatures with good agreement of the results.

Further general agreement has been found in the behavior of impaled cells of *V. macrophysa* in the course of continued study at Bermuda, except that these cells show even larger polarization potentials, and recover more readily to a condition resembling the "constant" state of intact cells. These are reserved for discussion in a later paper.

As before, it is a pleasure to acknowledge the generous co-operation of the Carnegie Institution of Washington in offering the facilities of its laboratory at Tortugas.

### III

This section describes the characteristic values of potential difference naturally existing across the protoplasm of *Valonia ventricosa*. As indicated in a preliminary report,<sup>7</sup> these differ somewhat from those of *V. macrophysa* as described by Osterhout, Damon, and Jacques.<sup>3,4</sup> Theoretically the values of bioelectric potential, developed by the protoplasm in contact with different solutions, should have a close relation to the polarization potentials elicited by the flow of current to or from such solutions. This expectation has been justified in the correlation found between several types of polarization responses and the bioelectric potential which accompanies them.

<sup>7</sup> Blinks, L. R., *Carnegie Inst. Washington Year Book*, 1928-29, 28, 277.

We may indicate five typical stages of the bioelectric potential difference:

(1)

Initial mechanical shock—P.D. 5 mv. or less (inside positive).

This stage follows immediately after impalement and lasts from a few minutes to several hours, depending apparently on the degree of injury sustained. The earliest measurable P.D. (some 10 seconds after impalement) is about 5 mv.; this usually drops within a few minutes to nearly zero. There is then a slow recovery of 1 or 2 mv. per hour, this process passing into stage (2) without apparent break. But in occasional cases there may be a complete reversal of P.D. to about 30 mv. (*outside positive*). This usually occurs promptly after impalement, lasts but a few minutes and is as promptly recovered from. It seems to occur most frequently in cells which have already reached the "constant" state before impalement, and it is really a momentary example of stage (4), probably elicited by the breakdown of the outer layer (*X*) of the protoplasm.<sup>4</sup> As *X* recovers or *Y* in turn breaks down, the P.D. returns to zero and the remaining slow recovery process ensues.

It may be noted that the potential differences given for stage (1) are in good agreement with those observed by Taylor and Whitaker<sup>8</sup> soon after the insertion of a micro-electrode into the vacuole of *Valonia ventricosa*. They state that there was "a relatively rapid lowering of the potential difference, which was then followed by a gradual rise to a maximum . . . . Although the amount of voltage varied for different cells, we have estimated a mean value of the potential difference between the cell sap of *Valonia* and sea water to be about 0.002 volt. The charge of the cell sap of *Valonia* was plus with reference to sea water." Had they followed the process longer there can be no doubt the P.D. would have been found to rise to values typical of stage (2). The necessity of such long waits for impaled cells to become stabilized was insufficiently realized for a long time, and probably also accounts for the low values (1 or 2 mv.) reported by Osterhout,<sup>9</sup> who first measured the P.D. in this manner in *Valonia*, and by Jost<sup>10</sup> who repeated them. Umrath<sup>11</sup> has recently stated that his earlier reports for *Nitella* were likewise too

<sup>8</sup> Taylor, C. V., and Whitaker, D. M., *Carnegie Inst. Washington Year Book*, 1925-26, 25, 248.

<sup>9</sup> Osterhout, W. J. V., *J. Gen. Physiol.*, 1925-26, 7, 561.

<sup>10</sup> Jost, L., *Sitzber. Heid. Ak. Wiss.*, 1927, Abh. 13, 1.

<sup>11</sup> Umrath, K., *Protoplasma*, 1930, 9, 576.

low for the same reason. This need of recovery was first pointed out by Osterhout, Damon, and Jacques.<sup>3</sup> In early experiments of the author to measure the electrical resistance of impaled cells, great ambiguity was met through similar neglect of the time factor, and the method was temporarily abandoned.

## (2)

The stage of recovery and normal potential: P.D. from 8 to 25 mv. with an average of 15 mv. (inside positive).

This is reached within several hours to 2 days after impalement, and persists through the life of the cell, with a gradual decrease of value.

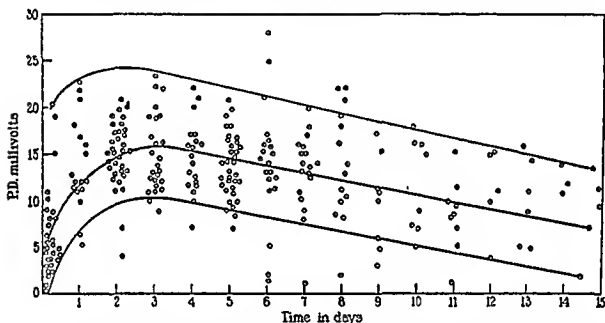


FIG. 2. Composite graph of the P.D. measured across the protoplasm during the life of 34 impaled cells of *Valonia ventricosa* in sea water. Each point is a single determination. The outer lines roughly enclose nearly all the scattered values, and the median line is drawn freehand to represent an average value. The P.D. is that of the vacuole, which is positive in the electrometer circuit to the sea water.

Fig. 2 gives a composite picture of the course of these values for a representative group of cells. The variations from day to day might be several millivolts, but in general a given cell had a more or less constant P.D., and those which were apparently most healthy (*i.e.*, dark green, turgid, and shiny) gave the highest values.

It should be noted that this P.D. is in the same direction as that of *V. macrophysa* similarly impaled and immersed in sea water, but has a normal value from 2 to 3 times as great. This is a specific difference, which if connected only with the higher potassium content of the sap

of *V. ventricosa*, must argue *against* a high mobility of  $K^+$  in protoplasm, at least at the inner surface, since the P.D. is in the wrong direction, the positive current tending to flow from water *inward* to the sap. The difference can probably be explained on the basis of 3 layers in the protoplasm.<sup>4</sup>

While stages (1) and (2) are the only ones necessarily passed by the living impaled cells, there are two other stages which temporarily be produced by special causes, and have importances to the polarization phenomena. These are:

(3)

A stage of increased P.D. (inside positive), produced by the immersion of various solutions. The highest of these measured was

a) 100 millivolts (inside positive), produced by the immersion of the cell in artificial sap (such as employed in the capillary tube<sup>5</sup>) is almost instantly attained, remains from 1 to 5 minutes, and then more slowly falls away to 5 mv. or less (inside positive). So the initial rise is only to 40 or 50 mv. There has not yet been the secondary rise shown by *V. macrophysa* at its first contact with its own sap, and this behavior more resembles the succeeding imbibitions of sap to that species.<sup>4</sup> The effect may be produced temporarily in *V. ventricosa* if the cell is returned to sea water for several minutes between exposures to sap. The rise of P.D. might be attributed to the high mobility of the  $K^+$  ion in the *outer* surface of the protoplasm; its succeeding fall to the total imbibition of the latter with KCl, thus leveling the gradient of  $K^+$  concentration across the outer surface. The plasmic asymmetry might thus be said to exist after the exposure of the cells to sea water, but to disappear upon their imbibition with sap. According to Damon's explanation<sup>4</sup> for the shape of curves in successive applications of sap to *V. macrophysa*, the middle layer (W) of protoplasm in *V. ventricosa* might further be considered always well imbibed with KCl. 0.6 M KCl acts much like sap in the experiments.

b) In addition to the large temporary increase of P.D. produced by immersion in sap, other, smaller, increases were produced by immersion of the cells in diluted sea water (1/10 sea water to 9/10 isotonic glycerin) or in 0.6 M  $MgSO_4$ . In each case the P.D. rose to 35 or 40 mv.

positive) and remained up for half an hour or longer. This increase is very much like the effect of dilute sea water on *V. macrophysa*.<sup>12</sup> The direction of this increase argues for a higher mobility of some anion, presumably  $\text{Cl}^-$ , in the inner surface of the protoplasm, since the gradient is greatly increased by these treatments. Immersion of the cells in 0.6 M NaCl promptly brings the P.D. nearly to zero, where it remains, and this could be ascribed to the abolition of the  $\text{Cl}^-$  gradient.

We now come to a fourth typical and very interesting stage:

(4)

Reversed potential: P.D. to 50 mv. (*outside* positive).

This frequently occurs after a long exposure to dilute sea water or  $\text{MgSO}_4$  solution, especially just after the cells are re-immersed in ordinary sea water. The change to this level is very rapid, sometimes being completed in a few seconds. The recovery from it is usually somewhat slower, and its total duration is seldom more than 5 or 10 minutes. Its occurrence shortly after impalement in some cells has already been noted under stage (1). It may also be elicited after very large current flows, *either* of inward or of outward current. For the latter it is not in the direction of a counter E.M.F. and therefore is distinct from the polarization response. Its independence of the current direction, and its production under a variety of circumstances, suggests rather that it is in the nature of an "alteration" such as the temporary destruction of the outer protoplasmic layer ( $X$ )<sup>4</sup> (or the reduction of its E.M.F.), leaving the outwardly directed E.M.F. of  $Y$ . This alteration is not permanently injurious to the cell, and is indeed characteristically found in those cells most nearly "normal" or "constant" in their apparent resistance. Thus it is most frequently seen just after "constant" cells are impaled, and then again toward the end of stage (2) when the cells have recovered from the injury of impalement. The ease of its production may then actually become troublesome in the measurement of bioelectric potentials.

<sup>12</sup> Damon, E. B., and Osterhout, W. J. V., *J. Gen. Physiol.*, 1929-30, 13, 445.

## (5)

The abolition of potential: (P.D., 1 mv. or less).

This stage is reached on the death of the cell even before the protoplasm is obviously disintegrated. It may be produced by long applications of single salt solutions, or by heat, or by chloroform and other toxic agents. It is of no particular biological interest therefore, but serves as a check on the electrodes and on the experimental technique.

## IV

We may now turn to the phenomena caused by the flow of current at these different stages.

Stage (5) has already been referred to in the discussion of methods, and serves as a convenient check upon the constancy of the electrodes and of the capillary resistance. In impaled cells just killed by chloroform, as much as 5 volts may be applied to the electrodes, and an approximately constant resistance measured during as long as a half hour of current flow in either direction. Neither electrodes, capillary, dead protoplasm nor cell wall introduce any significant change of resistance during this time. This shows that the heating effect of the current is negligible, and that migration of sea water into the vacuole is small.

In stage (1) are found rather similar conditions, except that the resistances tend to be slightly higher than those of the blank capillary before its insertion, and there is a feeble reaction, shown in the detector as a small back E.M.F. at the break of the current (of either direction). Occasionally there is a slightly higher apparent resistance to a small outward current than to an inward current, especially in cells where the reversion to stage (4) occurs. In general the effective resistance of the freshly impaled cells is so low as to be negligible at all current densities. This unexpected result in the early experiments of the author led to the temporary abandonment of the capillary method, since it could not be reconciled with the high effective resistance of the same cell measured just before its impalement. The low resistance is almost certainly due to leaks around the insertion of the capillary, but also to more profound changes in the entire protoplasmic surface. The nature of these changes begins to appear in the recovery process.

Stage (2). If now, after the cell had been impaled several hours, or as much as a day, a small potential (0.25 to 0.5 volt) was applied to the electrodes, the behavior was even more like that of stage (5) or a dead cell. The small reaction (back E.M.F.) shown in stage (1) had usually disappeared, and the resistance was essentially equal to that of the blank capillary before its insertion (or to the alternating current resistance measured at the moment). When the potential was increased in a direction to carry the positive current *outward* (from sap to sea water) across the protoplasm, this low resistance was maintained, even up to currents of 100 microamperes (caused by the application of 5 volts to a capillary of 50,000 ohms resistance).

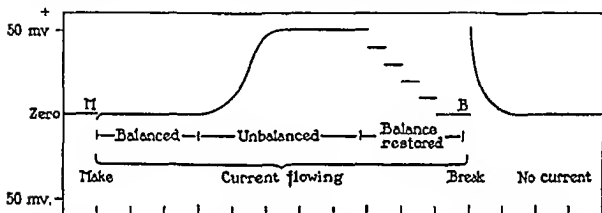


FIG. 3. Tracing from string galvanometer record showing deflections of the bridge detector during the flow of current inward across the protoplasm of an impaled cell of *Valonia ventricosa*. The upward movement is due to a rise of resistance, or to the appearance of an E.M.F. opposed to the applied potential. After the rise has occurred  $R$  is increased in 5 steps of 1000 ohms each to bring the detector to zero. At break there is a momentary upward movement due to the back E.M.F.  $R = 45,000$  ohms, later increased to 50,000 ohms. Applied potential, 1 volt. Detector sensitivity in open circuit as shown. Time marks, 1 second.  $M$ , make;  $B$ , break. (P.D. of cell was compensated to zero before the record.)

Only when the current was reversed and passed *inward* across the protoplasm from sea water to sap was there a change of the apparent resistance. At slightly higher voltages, between 0.5 and 2 or more volts in these early stages, this change occurred rather suddenly (Fig. 3). At the critical voltage there was at first no response to the flow of current, but in from 1 to 20 seconds there began a rise of apparent resistance, at first slow, then very fast, then slower again, sometimes carrying the image of the galvanometer fiber out of the



field. When this was brought back to the center by balancing the decade resistance box of the adjacent bridge arm, a steady state was now maintained as long as the potential was applied to the bridge. The amount of the rise varied from 1 or 2 per cent up to 10 per cent or more of the capillary resistance, and sometimes as much as 10,000 ohms additional resistance was required in the decade box to balance this apparent rise. What now occurs at the break of current, when the potential is removed from the bridge input terminals? In an ordinary resistance balance, through a wire resistance or through a blank capillary, the galvanometer image being brought to zero remains there

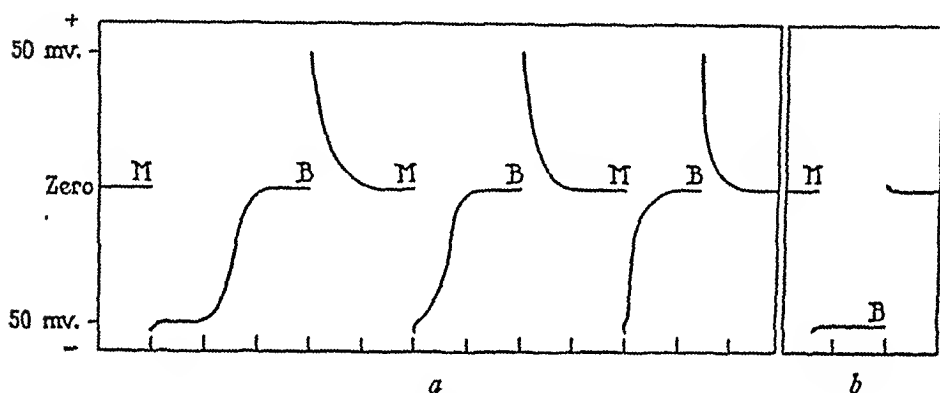


FIG. 4. Tracing from string galvanometer record showing deflections of the bridge detector during applications of 1 volt to the cell of Fig. 3.  $R = 50,000$  ohms throughout. *a*, inward current, 3 applications. *b*, outward current. Detector sensitivity in open circuit as shown. Time marks, 1 second. *M*, make; *B*, break. (P.D. of cell was compensated to zero before the record.)

when the potential is removed from the bridge. But in this case the image deflected greatly at the instant the outside potential was removed, and then returned slowly to zero again. Herein the cell resembled a platinum electrode, or a large condenser, and the deflection at break was due to the accumulated charge, or polarization potential. This is shown in Fig. 3, together with the preceding rise of apparent resistance and the balance to zero.

If, now, the decade box increment being retained and the bridge balance kept at the steady state value reached in Fig. 3, we apply the same critical voltage again, the record of Fig. 4 ensues. A rather long wait has followed the "break" of Fig. 3, so that the string image

is found now to be deflected approximately as far downward as it previously had risen upward. There is a delay of about a second at this level, and then the rise begins again, at first slowly, then more rapidly, finally flattening out at the center (zero) line representing the bridge balance. At removal of the potential from the bridge ("B") there is again an upward deflection, due to the back E.M.F. When this has returned fully to zero for about a second, the potential is again applied to the bridge and again there is a deflection downward, which is much more rapidly recovered from, but still shows the double inflection of the first curve. Again there is an upward deflection at

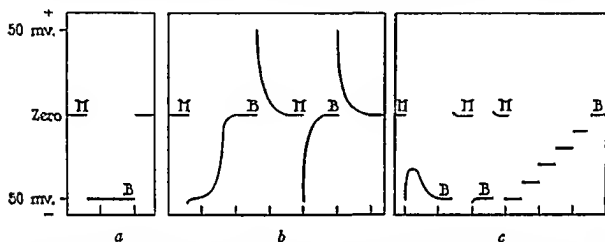


FIG. 5. Tracing from string galvanometer record showing deflections of bridge detector during applications of 1 volt to cell of Figs. 3 and 4, at a partially recovered state. *a*, Outward current; *b*, inward current; *c*, outward current. In the latter the first exposure causes a partial polarization, followed by a regression.  $R = 50,000$  ohms until the 3rd exposure in *c*, when it is reduced in 1000 ohm steps to 45,000 ohms. Detector sensitivity in open circuit as shown. Time marks, 1 second. *M*, make; *B*, break.

"break," returning smoothly to zero. A third application after another second's delay gives a smooth curve which has almost no hint of a double inflection and is nearly an image of the return curve at break. It now closely resembles the charge and discharge curves of a condenser connected in the bridge, or the polarization curves of a blank platinum electrode. It is evident that an effect of previous flow of current persists from one exposure to the next, and that there has been a progressive change in the response of the protoplasm suggesting that of a change from a reversible electrode to a polarizing

electrode. This however, holds only for *inward* currents, and if the current be reversed through the cell we obtain the record of Fig. 4*b*. The galvanometer image deflects immediately downward, and stays there as long as the current flows. The downward deflection is due to the maintenance of the bridge balance at the augmented value of  $R$  caused by the inward flow of current. If the balance be returned to that of the basal resistance  $S$ , the string image returns to zero, and there are no appreciable polarization phenomena either at make or break (*i.e.*, the record is essentially a straight line). The protoplasm is apparently a perfectly reversible electrode to an outward current.

This condition may prevail for many days after impalement. But sooner or later the protoplasm begins to polarize also to an outward current, and this is foreshadowed by the behavior shown in Fig. 5. The first section (*a*) repeats Fig. 4*b* for the bridge balance to the augmented state; there is seen to be no appreciable polarization with an outward current. The direction of the current through the cell was now reversed and the prompt polarization characteristic of the inward current was produced (Fig. 5*b*). The exposure was repeated, with even quicker response. Now the current was again quickly reversed, and Fig. 5*c* shows the response occurring with an outward current, followed by an immediate regression to the level of Fig. 5*a*. On break there is little or no back E.M.F., the image returning directly to zero without an excursion above the line. On the second application there is not even a temporary polarization, and the deflection is instant and complete. Finally the record shows the restoration of the bridge balance to the basal value ( $S$ ) in steps of 1000 ohms.

It is evident from this record that the protoplasm is still not ordinarily polarizable to an outward current, but may be made so temporarily. Either by the movement of ions out of the protoplasm into the vacuole, or by the alteration of the protoplasmic properties, the previous inward current has left an effect which persists a short time. The further recovery process in the course of stage (2) has the same effect as this flow of current and Fig. 6 shows the response given at a later time by a cell that is beginning to polarize normally with currents in either direction. Fig. 6*a* shows the prompt and symmetrical charge and discharge curves with the inward current. Fig. 6*b* shows nearly an equal response with an outward current, but there is a succeeding

regression which begins at a lower potential than with the inward current. This in general represents the behavior of the cells for the remainder of their life upon the capillary. They give good polarization with as low a potential as can be applied, and, for values up to about 0.5 volt (depending upon the resistance of the capillary) have a nearly constant apparent resistance with the current either direction. Above this value the apparent resistance may fall off, more rapidly with outward currents than with inward ones. The breakdown process is much the same in either case, being a regression of polarization following a preliminary rise, and simply occurs at lower

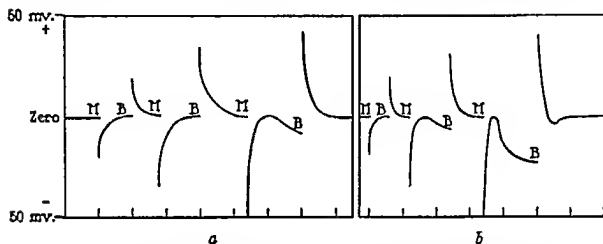


FIG. 6. Tracing from string galvanometer record showing deflections of bridge detector during applications of increasing voltages to an impaled cell of *V. ventricosa* which has reached the "constant" state. Applied potentials: 0.5, 1.0, and 1.5 volts, in the order given. *a*, Inward current; *b*, outward current. Detector sensitivity as shown (open circuit). Time marks, 1 second. *M*, make; *B*, break.

current densities in the outward direction than in the inward. In this the cells in the recovered condition of stage (2) resemble "constant" intact cells which have been chloroformed at one end, and are considered to have reached as nearly normal a state as the impaled cells ever do, having an apparent resistance that is independent of the applied potential up to the breakdown value.

In stage (3), the condition of increased P.D. across the protoplasm, the relations of stage (2) still hold quite well. Most remarkable of all, the polarization response obtained when the cell is immersed in artificial sap is about as great as when in sea water, and is characteristically even more rapid in its development, having a very abrupt

rise. After the exposure to sap has been continued for some time the polarization becomes less, and eventually may even be larger with an outward current than with an inward one. But for as long as half an hour after some cells have been immersed in artificial sap, their polarization response remained large and their apparent resistance as high as in sea water. It is difficult again to reconcile this result with a high mobility of  $K^+$  ion across the entire protoplasm, and it may become necessary to ascribe the effects to some unspecified ion, possibly

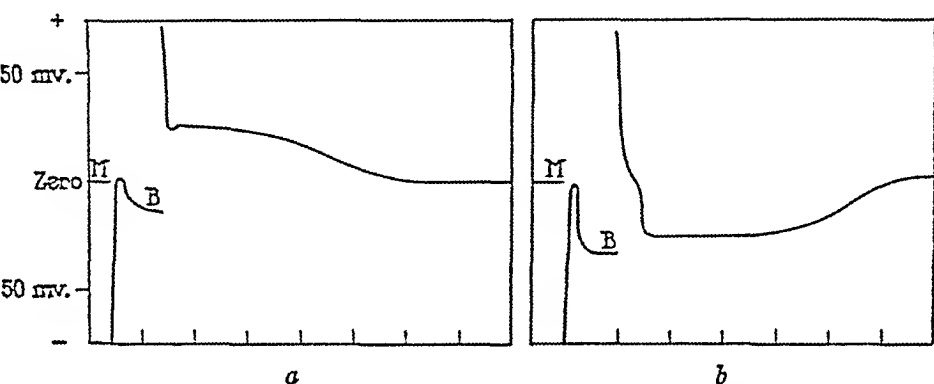


FIG. 7. Tracing from string galvanometer record showing deflections of bridge detector during applications of 3 volts to the cell of Fig. 6. *a*, Inward current; *b*, outward current (in *b* the cell is reversed in bridge). In each case the appearance of the polarization E.M.F. at break is succeeded by stage (4) with a reversed P.D. of about 50 mv. (outside positive).  $R = 38,000$  ohms; open circuit sensitivity of detector as shown. Time marks, 1 second. *M*, make; *B*, break.

existing only within the protoplasm, and not dependent upon the environment immediately supplied in the experiment.

Finally we come to the anomalies of stage (4). These consist both in its production, and in the polarization phenomena which occur when it is established. Its production mechanically (by impalement) and chemically (by dilute sea water, etc.) has been described above. Fig. 7 shows its production after the passage of large currents; in *a* after an inward, in *b* after an outward current. After the inward current, it has the same direction as the polarization potential and may normally add to the value of this. Certainly some frequent distortions of the return curve may be ascribed to a momentary production of this stage. But in the present case it forms a very

distinct level equal to about 50 mv., which persists for several seconds before returning to zero. In Fig. 7 *b*, after a large outward current has passed, there is first a large upward excursion due to the normal back E.M.F., but as this returns to zero there is a further movement, and the image goes below the zero line, again to an amount representing about 50 mv. This is now contrary to the polarization E.M.F., and is therefore in the same direction as the applied potential, which had driven the current *outward* across the protoplasm. The P.D. of stage (4) is thus in reverse of the normal potential and is *outside positive* to the electrometer. (In Fig. 7 *b* this P.D. appears in an opposite direction from Fig. 7 *a* because the cell has been reversed in the bridge.) Whether it is caused by the outward flow itself, or by the return current from the large polarization charge, is still uncertain. In either case it could be explained by the temporary alteration of the outer layer (X).<sup>4</sup>

While in these records the reversed P.D. of stage (4) lasted only a few seconds, in other cases it remained long enough for measurements of effective resistance to be made across the protoplasm while in this stage. As indicated before under stage (1), the usual polarization phenomena are now reversed, and the resistance appears to be greater to an outward current than to an inward one. This continues as long as the reversed P.D. remains, and then, as it begins to return to normal, polarization becomes more nearly equal in both directions and finally reaches that of the late stages of (2).

Study of the records has shown that the P.D. of stage (4) is frequently driven approximately to zero by an outward current. Since this upward movement is characteristic of a back E.M.F., it would therefore appear to be a polarization, and would be measured as an increased resistance. On the other hand, the P.D. of stage (4) is but little increased by an *inward* current, and there is almost no back E.M.F. developed in excess of the existing P.D. (which, of course, is taken as the null point, either balanced out or used as a pseudo zero). Thus an apparent polarization of as much as 40 or 50 mv. to an outward current may really be due to the destruction of so much P.D. This illustrates a pitfall inherent in these measurements, which may have to be taken account of in other biological "polarizations." That it is not entirely responsible for the total polarization to an outward current

has, however, been satisfactorily shown in *Valonia macrophysa*, and will be considered more fully in a paper dealing with that species. Since an increased polarization to an outward current sometimes appears during the exposure of cells to artificial sap, when there is no reversal of P.D. across the protoplasm, further interest is added to its study.

One question may remain concerning the recovery of impaled cells to a "constant" state. May not the polarization apparently occurring with currents directed outward really be due to their passage inward across a film of protoplasm formed over the orifice of the inserted capillary? Aside from the difference of constant intact cells which have been chloroformed at one end, and which show much the behavior of recovered impaled cells, there is the great difference of current densities between the point of the capillary where the current enters the vacuole and over the general surface by which it leaves. The area of protoplasm formed over the end of the capillary could not be more than 1 mm.<sup>2</sup> while that of the cell surface is usually from 2 to 3 cm.<sup>2</sup>—a more than thousandfold difference. It is practically assured therefore that the relatively immense current density—a thousandfold greater—across the smaller protoplasmic film, if any, would break this down and render its resistance and reactance negligible. A ten to hundred-fold increase is sufficient to do this on the remaining surface, as will be shown in a later paper dealing with injury and recovery. There is also the fact that such a protoplasmic plug would be bathed on its outer surface with artificial sap from the capillary, which is quite sufficient to destroy the protoplasmic properties of normal cells after exposures of a day or two.<sup>9</sup>

Still better evidence that such a film is not concerned is fortunately available, due to the straight open tubes employed in connection with the capillaries. With cells which had recovered to the "constant" state a fine platinum wire (attached on the end of a glass rod) was carefully inserted from above into the capillary, and a short distance into the vacuole. This was moved up and down gently and must have broken any plug which had formed. When the measurements were now repeated immediately after this probing the polarization effect was the same as before, and perfectly representative of the "constant" state as defined.

### V

It has already become sufficiently evident that the apparent rise of resistance in the impaled cells is not real or ohmic, but is due to a polarization potential. This, decreasing the current through the system by opposing the applied potential, is therefore measured as increased resistance. The reality of this back E.M.F. is evident from the string galvanometer records, and its responsibility for practically

all of the apparent resistance rise is shown by the essential constancy of the alternating current measurements throughout the life of the impaled cells, independent of their effective resistance to direct current.

This being so, the value of the polarization potential (in millivolts) becomes of more interest than the apparent resistance rise as measured in ohms. Several ways of determining this—by direct reading in a calibrated electrometer, by compensation with an opposed series potentiometer, or by measurement of the galvanometer deflection at the instant of break, as recorded photographically—have been employed, and will be reported upon in the paper dealing with *V. macrophysa*. These methods all agree well with the calculation of the back E.M.F., ( $P$ ), from the known values of the applied potential ( $V$ ), basal resistance ( $S$ ), and apparent resistance ( $R$ ), in which form the data for *V. ventricosa* were mostly recorded.

Thus in Fig. 3 where

$$V = 1.0 \text{ volt}$$

$$S = 45,000 \text{ ohms and}$$

$$R = 45,000 \text{ ohms (at the initial balance),}$$

there is at first no back E.M.F. and hence no deflection of the detector. As the back E.M.F. builds up, the detector deflects upward, and reaches an apparent value of approximately 50 mv. Since the sensitivity of the detector to a P.D. in the cell is reduced one-half with the bridge closed,<sup>2</sup> this P.D. is therefore about 100 mv. The polarization potential appearing at "break" is also seen to go momentarily to this value.

When now the bridge was balanced to the augmented steady state, in the five steps of 1000 ohms each, shown in Fig. 3.,

$$V = 1.0 \text{ volt}$$

$$S = 45,000 \text{ ohms, but}$$

$$R = 50,000 \text{ ohms}$$

By the equation

$$P = \frac{R-S}{R} \cdot V$$

the polarization potential  $P = \frac{50000}{50000} \cdot 1.0 = 0.1 \text{ volt or } 100 \text{ mv.}$ , which agrees with the directly observed potential.



mv. can effectively reduce to nearly zero a current through the protoplasm when 50 mv. is applied to an intact cell, such a back E.M.F. developed by the same initial current density in an impaled cell can only partly counteract a potential of one-half volt applied through the capillary. Thus a much larger current will continue to flow in the latter case than in the former, and to these differences of continued current density may be attributed some of the differences found between intact cells and impaled cells. The study of *V. macrophysa* has been particularly directed toward these differences, and the polarization potential will there be further considered in relation to the current density across the protoplasm.

The *quantity* involved in these effects, as distinct from the *intensity* (E.M.F.) of polarization emphasized in this paper, is the *capacitance* of the protoplasm in its function as a condenser acquiring a charge during a flow of current. It early became evident that the capacitance of *Valonia* cells is very large, *i.e.*, the *quantity* of electricity taken to charge them to a given potential is large and hence a long flow of current is required. Otherwise the records of charge and discharge could not be taken with a (relatively) slow-period instrument such as a string galvanometer. The fact that this instrument gives good records of these curves at quite ordinary camera speeds (2 cm. per second) has greatly simplified their study. The time relations will be considered in a forthcoming paper along with the closely related alternating current measurements. But an indication of the quantities involved is given in such curves as are shown in Figs. 3, 4, 5, and 6. The time course of these is often a second or more (as compared to one or two *thousandths* of a second in human skin<sup>13</sup>) and the time constant  $\tau$  (time for the potential of any part of the curve to change by  $\frac{1}{e}$  of its value) may be 0.2 or 0.3 second. Since (when  $\tau$  is in seconds,  $r$  in ohms, and  $c$  in farads)  $\tau = r c$ ; and  $r$ , the total bridge resistance through which the potential is discharged, is usually not over 100,000 ohms,  $c$ , the effective capacitance of the protoplasm, is of the order of 2 or 3 microfarads. Thus for a cell of about 1 cm. diameter and some 3 cm.<sup>2</sup> surface, this is as high as 1 microfarad per cm.<sup>2</sup>

It is interesting to note that capacitances of this general magnitude

<sup>13</sup> Hozawa, S., *Arch. ges. Physiol.*, 1928, 219, 111.

were also found at the lower frequencies of alternating current, in experiments with *Valonia* made several years ago,<sup>14</sup> and in later measurements to be published shortly. The figure is also in good agreement with that for red blood corpuscles ( $0.81 \mu F/cm.^2$ ) published by Fricke;<sup>15</sup> and, less closely, with that of McClendon<sup>16</sup> ( $9 \mu F/cm.^2$ ) for erythrocytes; and of Cole<sup>17</sup> for *Arbacia* eggs ( $1.02 \mu F/cm.^2$  at 350,000 cycles, or  $18 \mu F/cm.^2$ , extrapolated to 1000 cycles).

#### SUMMARY

Electrical resistance and polarization were measured during the passage of direct current across a single layer of protoplasm in the cells of *Valonia ventricosa* impaled upon capillaries. These were correlated with five stages of the P.D. existing naturally across the protoplasm, as follows:

1. A stage of shock after impalement, when the P.D. drops from 5 mv. to zero and then slowly recovers. There is very little effective resistance in the protoplasm, and polarization is slight.

2. The stage of recovery and normal P.D., with values from 8 to 25 mv. (inside positive). The average is 15 mv. At first there is little or no polarization when small potentials are applied in either direction across the protoplasm, nor when very large currents pass *outward* (from sap to sea water). But when the positive current passes inward there is a sudden response at a critical applied potential ranging from 0.5 to 2.0 volts. The resistance then apparently rises as much as 10,000 ohms in some cases, and the rise occurs more quickly in succeeding applications after the first. When the potential is removed there is a back E.M.F. displayed. Later there is also an effect of such inward currents which persists into the first succeeding outward flow, causing a brief polarization at the first application of the reverse potential. Still later this polarization occurs at every exposure, and at increasingly lower values of applied potentials. Finally there is a "constant" state reached in which the polarization occurs

<sup>14</sup> Blinks, L. R., Thesis deposited in the library of Harvard University, 1926. Abstract in Summaries of Theses (1926), Cambridge, 1930, 10.

<sup>15</sup> Fricke, H., and Morse, S., *J. Gen. Physiol.*, 1925-26, 9, 137.

<sup>16</sup> McClendon, J. F., *J. Biol. Chem.*, 1926, 69, 733.

<sup>17</sup> Cole, K. S., *J. Gen. Physiol.*, 1928-29, 12, 37.

with currents of either direction, and the apparent resistance is nearly uniform over a considerable range of applied potential.

3. A state of increased P.D.; to 100 mv. (inside positive) in artificial sap; and to 35 or 40 mv. in dilute sea water or 0.6 M  $\text{MgSO}_4$ . The polarization response and apparent resistance are at first about as in sea water, but later decrease.

4. A reversed P.D., to 50 mv. (outside positive) produced by a variety of causes, especially by dilute sea water, and also by large flows of current in either direction. This stage is temporary and the cells promptly recover from it. While it persists the polarization appears to be much greater to *outward* currents than to inward. This can largely be ascribed to the reduction of the reversed P.D.

5. Disappearance of P.D. caused by death, and various toxic agents. The resistance and polarization of the protoplasm are negligible.

The back E.M.F. of polarization is shown to account largely for the apparent resistance of the protoplasm. Its calculation from the observed resistance rises gives values up to 150 mv. in the early stages of recovery, and later values of 50 to 75 mv. in the "constant" state. These are compared with the back E.M.F. similarly calculated from the apparent resistance of intact cells.

The electrical capacitance of the protoplasm is shown by the time curves to be of the order of 1 microfarad per  $\text{cm}^2$  of surface.

## ANNOUNCEMENT

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# ELECTROKINETIC PHENOMENA. III

## THE "ISOELECTRIC POINT" OF NORMAL AND SENSITIZED MAMMALIAN ERYTHROCYTES

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*(Accepted for publication, July 24, 1930)*

### I

#### INTRODUCTION

Kozawa (1) observed in 1914 that the addition of sufficient acid to mammalian red blood cells brought them to an isoelectric point at a pH which was characteristic for the cells of each of the animals studied. The amount of acid needed, however, was not constant for any particular animal, but decreased with the time the cells were permitted to stand in contact with the acid. It is evident, therefore, from these early experiments that time as well as acidity was influential in producing a reversal of sign of charge. These facts have not hindered a more or less general acceptance of the isoelectric point of red blood cells as a physical constant, apparently in much the same sense that it exists for the proteins. The reversal by hydrogen ion of the sign of charge of surfaces coated with proteins like egg albumin, and of globulin particles, occurs independent of time, with equilibrium, as far as our present technic permits, occurring instantly and remaining constant. The isoelectric point of a system is not significant in a simple sense unless it remains constant with time. If it does not remain constant, the variation points to a change in the surface or substance investigated and the "isoelectric point" does not represent a physical constant characteristic of the system. If the point of equilibrium is unrelated to the original system, except in a secondary way, the isoelectric point determined may be the resultant of many forces whose resolution can hardly be determined by the simple determination of reversal of sign of charge.

Coulter (2) (1920) studied the migration of sheep's red blood cells washed with saline solution and suspended in a saccharose solution made acid with HCl, acetate, or phosphate buffers. He used a macroscopic method which involved long experimental periods before readings of mobility could be made. Coulter concluded from his data:

" . . . that the direction and rate of movement in the electric field of both normal and sensitized red blood cells is a function of the hydrogen ion concentration. At concentrations less than pH 4.6 the charge carried is negative and increases in amount with the alkalinity; pH 4.6 represents the isoelectric point; at concentrations greater than pH 4.6 the charge carried is positive and increases in amount with the acidity."

"A comparison of the two curves<sup>1</sup> shows that on the alkaline side of the isoelectric point the charge of normal cells is greater and increases more rapidly with alkalinity than the charge of sensitized cells."

Shortly after the work of Coulter it was found by Eggerth (3) that the migration velocities of *B. coli* suspended in buffer mixtures commonly used for acid agglutination underwent a change in mobility, the organisms becoming less negative. He concluded that these changes were due to the extraction of a protein which was combined at the interface of organism and medium. In a second paper Eggerth (using a microscopic method to determine mobility) pointed out that similar changes occurred in erythrocyte suspensions.

Eggerth states, on the one hand:

"Human and sheep erythrocytes when placed in 0.01 N phthalate buffer solutions at reactions more acid than pH 5.2 undergo a progressive change in potential, becoming less negative or more electropositive. This change usually occurs within two hours at ordinary room temperatures. . . . This change is primarily due to the liberation of hemoglobin from the cells."

On the other hand, after thus showing that red cells were destroyed in acid solutions and that their mobilities varied with time, he states:

"The isoelectric point of erythrocytes in the absence of salt or in the presence of salts having both ions monovalent occurs at pH 4.7. This confirms the observations of Coulter."

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<sup>1</sup> Curves for normal and sensitized cells.

Netter (4) has made a careful investigation of the electrophoretic mobilities of horse and ox cells in acid solutions to determine the isoelectric points. The isoelectric point of washed and unwashed horse red cells was at pH 4.7 in M/40 acetate. Washed ox cells were found to be isoelectric at pH 4.3 in M/40 acetate.

These data of Netter are concordant with those of Eggerth, not only in the existence of an isoelectric point for the red cells investigated but also in the fact that near the isoelectric point some time passed before "equilibrium" was obtained. He states:

"Das Potential stellt sich nach Uebertragen in die Loesung im allgemein schnell ein und bleibt constant, nur in der Gegend des isoelektrischen Punktes is das endgultiges Potential erst nach 2 Stunden spätestens erreicht; *alle Messungen wurden daher nicht vor 2 Stunden nach dem Ansetzen der Suspensionen begonnen.*"

Netter cites further the data of Eggerth, mentioning the effect of the products of red cell destruction.

Eagle (5) (1930) states:

"Similarly, sensitization changes the cataphoretic potential of red cells; however, according to Coulter and confirmed by us, the cataphoretic isoelectric point remains at its *normal* value (pH 4.7)"

(the italics are ours). Further on this author writes:

"Red cells<sup>2</sup> both normal and sensitized have a minimum velocity at about pH 4.7: in more acid reaction there is hemolysis, with currents making readings impossible."

Fig. 1 summarizes graphically some of the pertinent results of Coulter, Eggerth, Netter and Eagle. All of the data of these authors are early or late *equilibrium* values of the isoelectric point obtained in *acid* solutions incidental to the presence of the products of hemolysis.

The existence of an isoelectric point for the mammalian red cell will be here more closely scrutinized in the light of these previous investigations and of our own. It will be shown that it is, at present, more in keeping with experiment to believe that normal red cells have no known values of pH for their isoelectric points, when suspended in uni-univalent electrolytes. While reversal of sign of charge may occur,

<sup>2</sup> The animal from which the red cells were obtained is not given.



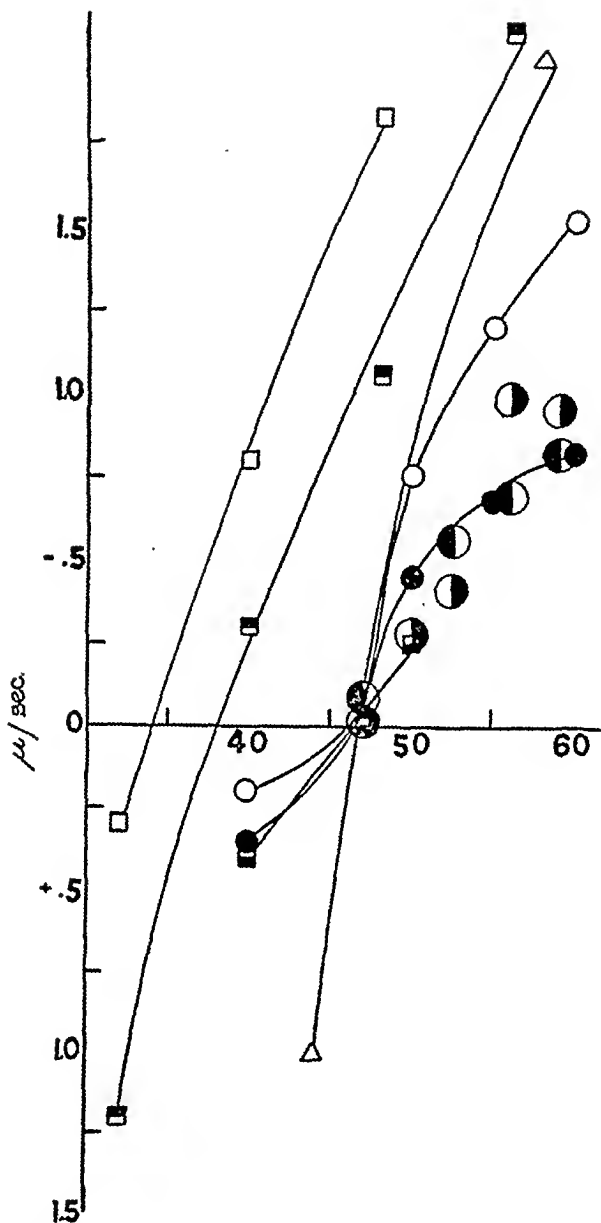


FIG. 1. The relationship between pH and electric mobility according to various authors:

- Coulter; normal cells (relative units).
- Coulter; sensitized cells (relative units).
- Eggerth; zero time
- Eggerth; 1.5 hours
- Eggerth; rabbit cells in 0.01 N NaCl.
- △ Netter; washed horse red cells in sugar buffered by N/40 acetate.
- Eagle; normal red cells in NaCl solution buffered by acetate (relative units).
- Eagle; sensitized red cells.

For further description, see text.

it will be shown that its demonstration is most unlikely with intact, normal human and sheep red blood cells.<sup>3</sup>

## II

### *Methods*

The measurements were performed in the modification of the Northrop-Kunitz cell described previously (6). Measurements in acid solutions were usually made only at one level (0.2) in the cell, since destruction of cells occurred in acid solutions so rapidly that further measurements were inconvenient. At the level, 0.2, the speed of the particle relative to the liquid is measured.

*All measurements were made within two minutes of the time that acid was added to the system.*

The description of the experimental procedure is best given with each experiment, in the next section.

The red cell suspensions were buffered sufficiently to prevent the occurrence of significant changes in pH due to hemolysis. No experiments with saccharose or other sugar were performed, for the following reason. Change in ionic strength is known to influence greatly the solubility and state of proteins. More normal conditions may be obtained for proteins existing at a phase boundary by not varying ionic concentration or species unnecessarily. If the ionic strength is varied it is evident that a new variable is introduced.

Hydrogen ion activity was determined by the quinhydrone electrode. All measurements are referred to pH 4.62 as the pH of Michaelis acetate standard.

## III

### *The Isoelectric Point of Normal and Sensitized Cells*

*Experiment I.—Unwashed Human Red Blood Cells.* Human blood was drawn up into the stem of a white blood cell pipette from a pricked finger and mixed directly with 100 cc. of 0.85 per cent sodium chloride solution buffered by N/50 sodium acetate-acetic acid solutions. Measurements of mobility were made within 2 minutes of preparation of the suspension.

Note that reversal of sign of charge did not occur; nor was there an appreciable decrease in mobility with decrease of pH. The fact that the mobility at pH 3.9 was so close to that observed at pH 5.7, where practically no destruction of red cells occurs within the short interval,

<sup>3</sup> Preliminary data have also been obtained for rabbit red cells. The conclusions are similar.

indicates that these mobilities are related more to the normal chemical constitution of the red cell than to complex phase boundary conditions occurring in acid media.

*Experiment II.—Washed Human Cells.* 5 cc. of human blood were poured into 25 cc. of 0.85 per cent NaCl and centrifuged. The cells were washed 6 times with 10 times their volume of 0.85 per cent NaCl containing N/100 sodium acetate. Measurements of mobility were made within 2 minutes after addition of acetic acid.

Table II confirms that data found in Table I.

TABLE I

| pH  | V                                       |
|-----|---|
|     | $\mu/\text{sec}/\text{volt}/\text{cm.}$ |
| 5.7 | -1.15                                   |
| 4.6 | -1.03                                   |
| 4.0 | -0.97                                   |
| 3.9 | -1.02                                   |

TABLE II

| pH   | V                                       |
|------|---|
|      | $\mu/\text{sec}/\text{volt}/\text{cm.}$ |
| 5.6  | -1.05                                   |
| 4.1  | -1.10                                   |
| 3.6  | -1.04                                   |
| 3.55 | -0.92                                   |

*Experiment III.—Washed Sheep Cells.* Fresh defibrinated sheep cells which had been in the ice chest for 12 hours<sup>4</sup> were washed 8 times with about 10 times their volume of 0.85 per cent sodium chloride solution containing N/50 sodium acetate. The acetate was added to keep the suspension alkaline. Suspensions of red cells were made up as in Experiment I and measurements were made within 2 minutes.

Because destruction of cells occurs with such rapidity in acid solutions, the slight diminution in mobility described in Table III cannot be unequivocally correlated with decreasing pH. The possibility

<sup>4</sup> No appreciable change in mobility occurs if horse red cells are kept in serum in the ice chest as long as 3 days.

remains that this slight but definite change may be due to adsorption of the products of erythrocyte destruction. This will be subsequently discussed in detail.

*Experiment IV (A).—Sheep Cells in the Presence of Rabbit Serum.* Sheep cells of Experiment III<sup>5</sup> were added to 50 cc. of 0.85 per cent sodium chloride containing, in addition, N/50 sodium acetate, and 1 cc. of washed<sup>6</sup> normal rabbit serum which had been heated to destroy complement. The sheep cells were allowed to

TABLE III

| pH   | V<br>$\mu$ /sec/volt/cm. | Remarks  |
|------|--------------------------|--|
| 6.45 | -1.16                    | In these acid solutions hemolysis was very rapid and at times complete within 2½ minutes |
| 6.26 | -1.03                    |  |
| 5.91 | -1.12                    |  |
| 4.73 | -1.01                    |  |
| 4.30 | -0.98                    |  |
| 3.72 | -0.97                    |  |
| 3.62 | -0.94                    |  |
| 3.60 | -0.88                    |  |

TABLE IV

| pH   | V<br>$\mu$ /sec/volt/cm. | Remarks   |
|------|--------------------------|---|
| 6.18 | -1.14                    | Control: No serum; pH 3.51, V = -0.75 $\mu$ /sec/volt/cm. |
| 4.25 | -0.97                    |   |
| 3.90 | -0.56                    |   |
| 3.67 | ±                        |   |

remain in contact with this solution for 15 minutes. Measurements of mobility were made within 2 minutes after addition of acid. See data in Table IV.

Table IV demonstrates that above pH 4.2 the presence of rabbit serum protein does not appreciably influence sheep red cell mobility. This is in harmony with our previous findings that human cells sus-

<sup>5</sup> Twelve hours later and washed twice with salt-acetate.

<sup>6</sup> This refers to a preliminary treatment of the "normal" rabbit serum with rabbit red cells to remove any amboceptor. This treatment does not remove an appreciable quantity of serum protein.

pended in isotonic media containing 1.4 per cent of rabbit serum retain their surface integrity and migrate about twice as quickly as rabbit cells suspended in the same medium (7). The interpretation of the fact that red cells are isoelectric at about pH 3.6 is difficult. Since this change in mobility occurs in a region where red cell destruction is extremely rapid, it seems reasonable to conclude that the drop in

TABLE V

| pH   | V<br>$\mu\text{sec/volt/cm.}$ | Remarks        |  |
|------|-------------------------------|----------------|--|
|      |                               | Controls<br>pH | Without serum<br>V<br>$\mu\text{sec/volt/cm.}$ |
| 5.50 | -1.00                         | 5.55           | -1.04  |
| 4.65 | -1.00                         | 4.65           | -0.91  |
| 4.60 | -0.95                         | 3.54           | -0.79  |
| 4.10 | -0.53                         |                |  |
| 3.74 | negative                      |                |  |
| 3.64 | positive                      |                |  |

TABLE VI

| pH   | V<br>$\mu\text{sec/volt/cm.}$ | Remarks        |   |
|------|-------------------------------|----------------|---|
|      |                               | Controls<br>pH | No serum<br>V<br>$\mu\text{sec/volt/cm.}$ |
| 6.5  | -0.58                         | 4.72           | -1.04                                     |
| 4.97 | -0.60                         | 3.5            | -0.89                                     |
| 4.70 | -0.45                         |                |   |
| 4.0  | -0.43                         |                |   |
| 3.6  | $\pm$                         |                |   |

mobility and attainment of an isoelectric state is determined by the presence of abnormal red cell surfaces as well as by the presence of serum protein. We believe, therefore, that the isoelectric point noted has no more significance than that which may be attached to those red cell systems described in the introduction.

*Experiment IV (B).—Sheep Cells in Presence of Rabbit Serum.* Experiment repeated in N/100 acetate buffer. See data in Table V.

The discussion following Table IV applies here.

*Experiment V (A).—Sensitized Sheep Cells.* The sheep red cells used in IV (B) were kept in the ice chest for 6 days. At the end of this time they were washed 8 times with about 10 times their volume of 0.85 per cent sodium chloride containing  $N/50$  sodium acetate. A very dilute suspension of the cells (about 1:2000) was permitted to remain about 15 minutes in contact with 24 cc. of physiological saline containing 1 cc. of an inactivated rabbit anti-sheep serum. At the end of this period a suitable mixture of electrolytes was added to make conditions identical with part (b) of this section. Measurements were completed within two minutes. See data in Table VI.

*Experiment V (B).—Sensitized Sheep Cells.* Experiment V (a) was repeated in  $N/100$  acetate buffer. See data in Table VII.

TABLE VII

| pH   | $\frac{V}{\mu\text{sec/volt/cm.}}$ |
|------|------------------------------------|
| 5.5  | -0.57                              |
| 4.35 | -0.60                              |
| 4.1  | -0.40                              |
| 3.9  | -0.28                              |
| 3.8  | negative                           |
| 3.7  | $\pm$                              |
| 3.6  | +0.24                              |

The data of Tables VI and VII demonstrate the well known effect of specific immune serum on red cell mobility. In one sense they confirm Coulter's statement that the isoelectric point of sensitized cells is not perceptibly different from that of normal cells. In view, however, of the fact that an excess of serum protein is present, and furthermore that protein of the serum alone is capable of inducing an isoelectric state near the pH noted, we feel we must ascribe the similarity found here for the isoelectric point of normal and sensitized cells to the same effect, possibly an injurious one, that has been described as occurring in solutions as acid as these. Indeed, the wide differences in mobility between normal and sensitized cells in the zones where injury to the cell surfaces is not marked, make it more likely that if isoelectric points with regard to hydrogen ion activity could be determined without red cell destruction, these points would be different.

What effect more powerful immune sera would have on the mobility is unknown for these conditions (See Fig. 2).

## IV

*The Adsorption of Gelatin by Red Cells*

It has been previously remarked on several occasions that horse red cells in (alkaline) serum gelatin gels do not adsorb gelatin in the same

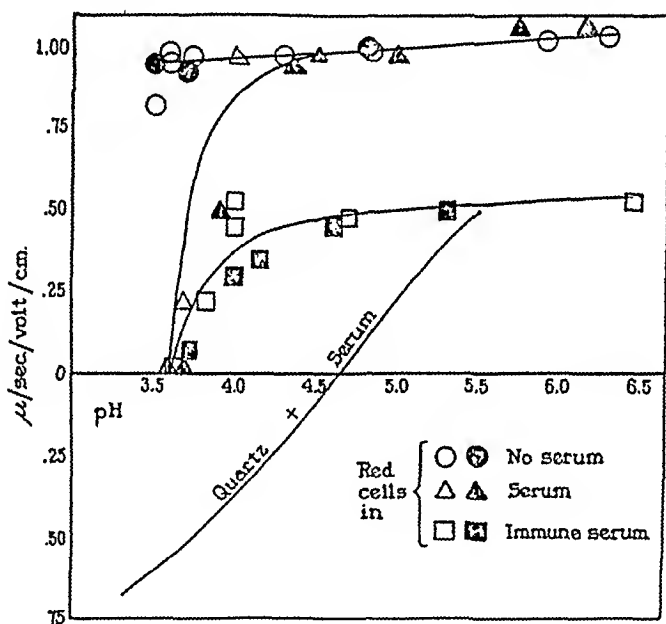


FIG. 2. A summary of the type of data obtained here for the electrophoretic mobilities of sheep red cells in 0.85 per cent NaCl buffered by acetate. Note that in the absence of serum under the experimental conditions noted in the text, the slope of the straight line describing the relationship between mobility and pH is very small. Serum has no demonstrable effect up to about pH 4.3. Immune serum changes the surface notably even above pH 4.3. Compare with Fig. 1. Quartz particles adsorb protein of the serum in characteristic fashion. (See: Abramson, H. A., *J. Gen. Physiol.*, 1929, 13, 169.)

way that quartz particles do (8). That is, in just the same way that horse red cells preserve their surface integrity in the presence of serum, so do washed red cells keep their specific electrophoretic mobilities in the presence of gelatin in phosphate buffer at pH 7.35.

We have seen in the preceding section that adsorption of some serum protein probably does occur in acid solutions. It was of interest therefore to observe the mobilities of washed red cells that had been in contact with gelatin, and studied in acid solutions in the presence of gelatin.

*Experiment VI (A).—Washed Sheep Cells (Table VIII).*

TABLE VIII

| pH   | $\frac{V}{\mu\text{sec/volt/cm.}}$ | Remarks  |
|------|------------------------------------|--|
| 3.61 | -0.87                              | Cells of Experiment II (b) in 0.2 per cent gelatin |

*Experiment VI (B).—Washed Human Cells (Table IX).*

TABLE IX

| pH              | $\frac{V}{\mu\text{sec/volt/cm.}}$ | Remarks   |
|-----------------|------------------------------------|---|
| 5.9             | -1.00                              | Cells of Experiment III kept 24 hours on ice, rewashed, and studied in 0.2 per cent gelatin |
| 4.33            | -1.06                              |   |
| 3.62            | -0.92                              |   |
| Control, quartz |                                    |   |
| 3.6             | +0.40 approx.                      |   |
| 5.7             | -0.25 approx.                      |   |

*Experiment VI (C).—Washed Sheep Cells (Table X).*

TABLE X

| pH   | $\frac{V}{\mu\text{sec/volt/cm.}}$ | Remarks   |
|------|------------------------------------|---|
| 4.00 | -0.93                              | Cells of Experiment IV (b) suspended in 0.2 per cent gelatin for 10 minutes and then examined |
| 3.76 | -0.75                              |   |
| 3.67 | -0.65                              |   |

The data in Tables VIII, IX, and X indicate that even though red cells in acid solutions may react with certain components of a medium containing normal serum, a similar reaction does not occur with gela-



tin. One is led to suspect that the reaction with the protein of the serum is not exactly as non-specific as the adsorption of serum protein by quartz. If so, the red cells should have adsorbed gelatin. Experiment VI (B) was made particularly striking by studying simultaneously quartz particles and red cells in the presence of gelatin. In these systems the red cells migrated to the anode and the quartz particles, because of their adsorbed protein film, migrated in the opposite direction.

It must not be thought that reversal of the sign of charge of red cells did not occur in the acid solutions. It did so repeatedly, but always after the 2-minute period of contact with the salt solution when the products of hemolysis were sufficiently concentrated to be an important factor. In *no case* was a positively charged red blood cell seen in acid solutions during the 2-minute period of observations.

It is of interest to describe how this reversal of sign of charge may occur. In Table X the cells suspended in the buffer at pH 3.67 were observed after the end of the 2-minute period during the very rapid hemolysis which occurs at this pH. Red cells which were seen migrating rapidly to the anode seemed to lose their hemoglobin suddenly and the pale "ghost" reverse its sign of charge migrating to the cathode.

## V

### *The Reaction of Sheep Cells with Normal and Immune Sera in Phosphate Buffer<sup>7</sup>*

An extensive series of measurements was made to investigate the effect of normal and immune rabbit sera on sheep cells. We shall not give the data in detail, for these experiments in general merely confirm what has hitherto been known qualitatively. They are of interest because of the precision which has been obtained in 0.85 per cent NaCl buffered by M/150 phosphate of initial pH 7.35, the medium in which all observations of mobility have been made, and because of the associated studies of hemolysis made in conjunction with changes in mobility. In the experiments described in general in Fig. 3, five different immune sera and four normal sera were employed. Curve 1 in Fig. 3

<sup>7</sup> When studying the effect of sera on cells, at least 1 hour's time elapsed between preparation of suspension and measurement.

was obtained by studying washed sheep cells in normal inactivated rabbit serum. It is evident that no change in mobility occurs up to about 1:50 of serum. Curve 2 has also been obtained with normal serum from another rabbit. At low serum dilution a definite lowering of electrophoretic mobility occurred. That this change was real, was evidenced by the fact that after addition of more sheep cells to the suspension, cells having two different mobilities were present. Curve 3 is a typical curve obtained with inactivated anti-sheep serum. Approximately the same value of mobility was obtained for all the

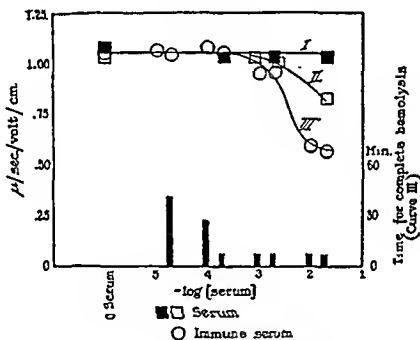


FIG. 3. See Part V of text. The upright black rectangles represent hemolysis times.

immune sera. Whether this is the lowest mobility obtainable cannot be decided upon from the shape of the curve, for the possibility exists that a more powerful anti-serum may be obtained than that employed.

The cells with which Curve 3 had been obtained were studied with a suitable dilution of complement to ascertain the relationship between the lowering of mobility and susceptibility to hemolysis by complement. It is to be noted that complete hemolysis on the addition of complement (0.03 cc. guinea pig serum to 1 cc. of a dilute red cell suspension) occurred in all except the highest dilution of serum, much before any perceptible change in mobility occurs. In other words,

sensitization to hemolysis of the red cell surface occurs without any change in mobility. In fact, hemolysis was just as rapid in the 1:5000 dilution as in the lower ones. At this dilution, as is apparent from the figure, there is no change in mobility, but hemolysis is rapid and complete. Since the mobility of the cell is in general determined by its surface constitution, very few active groups of the cell surface are changed for preparation of the cell for hemolysis incidental to the presence of complement. The lowering of the electrophoretic mobility does indicate a notable change in the surface make-up of the cell. As far as hemolysis requirements are concerned this change is secondary and must be associated with other immunological properties of the cell surface acquired incidentally to sensitization by a large quantity of amboceptor.

## VI

## SUMMARY

A survey of the published electrophoretic mobilities of certain mammalian red cells reveals that the isoelectric points accorded to these cells are the result of equilibria incidental to red cell destruction. The electrophoretic mobilities of normal washed sheep and human cells have now been studied in 0.85 per cent NaCl solutions from about pH 3.6 to 7.4. All measurements were made within 2 minutes of the preparation of the suspension of red cells. In no case was reversal of sign of charge observed under these conditions. Reversal of sign of charge occurred only after sufficient time had elapsed to permit sufficient adsorption of the products of red cell destruction. There is little change in mobility as the pH of the medium is decreased. Reversal of sign of charge does occur in the presence of normal and immune (anti-sheep) rabbit sera. The isoelectric point determined under these conditions does not appear to be connected specifically with the immune body but is perhaps associated with phenomena incidental to red cell destruction and the presence of serum. The characteristic lowering of mobility by amboceptor occurs, however, from pH 4.0 to pH 7.4. The curves of mobility plotted against pH for normal and for immune sera support the viewpoint that the identity of the isoelectric points for normal and sensitized sheep cells is not primarily concerned with the immune reaction. It is most unlikely that an "albu-

min" or a "globulin" surface covers red cells with a complete protein film. Although serum protein reacts with red cells in acid solutions, this is not demonstrable for gelatin. The lowering of mobility usually ascribed to anti-sheep rabbit serum may also occur, but to a lesser degree, in normal rabbit serum. This diminution of mobility is not, in the first place, associated with sensitization to hemolysis induced by complement. This supports the view that only a very small part of the red cell surface need be changed in order to obtain complete hemolysis in the presence of complement.

I am indebted to Professor Wm. J. Crozier for reading the manuscript and making many acceptable suggestions.

*Addendum.*—The viewpoint of McCutcheon, Mudd, Strumia, and Lucké (*J. Gen. Physiol.*, 1930, 13, 669) in regard to the isoelectric point of cells has recently appeared.

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# FACTORS INFLUENCING THE RESPIRATION OF ERYTHROCYTES

## I. PRIMITIVE AVIAN ERYTHROCYTES

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### INTRODUCTION

The function of the nucleus as the principal seat of the oxidative processes in the cell was first suggested by the investigations of Spitzer (1) upon the location of oxidizing enzymes. These observations were given considerable support by Lillie (2), who studied the problem with the aid of dyes that underwent color changes upon oxidation. He found that "the coloured oxidation products are seen to be deposited chiefly in and about the nucleus, especially at the surface of contact between nucleus and cytoplasm." These studies were followed up by Warburg (3) in a series of observations upon the comparative rates of reduction of the hemoglobin in the blood of mammals and birds, incubated for some hours in sealed vessels. He found that there existed a very marked difference between the two: the non-nucleated cells of the mammals had a comparatively small consumption of oxygen, whilst the nucleated ones in avian blood possessed a vigorous oxidative metabolism. He further showed that in both mammals and birds the oxidative metabolism proceeded at a much greater rate in blood which contained many young cells staining in a basophilic manner with methylene blue. In fact it appeared doubtful whether the fully developed normally staining mammalian red blood cells consumed oxygen to any appreciable extent. The whole of the slight oxygen consumption of the normal blood of adult mammals would seem to be due to the small proportion of young reticulated and basophilic cells ordinarily in the circulation.

The very large oxygen consumption of avian blood, especially after

hemorrhage when the proportion of primitive red cells in the circulation was high, led Warburg to a study of the factors which influence its metabolism. Many of the experiments were concerned with those factors that reduce or inhibit oxidation, and culminated in his well known theory of the importance of the part that structural integrity plays in the processes of cell oxidations (4).

The following experiments were carried out with the intention of determining rather more fully the effects that certain changes in environment produce upon the metabolism of the primitive avian red cells.

### *Methods*

The blood used in all the experiments was removed by cardiac puncture from fowls made anemic by intraperitoneal injections of phenylhydrazine hydrochloride. 10 cc. of a one half per cent solution in normal physiological saline was given to each bird. The blood was used from the third to the seventh day following the injection, and the removal, either daily or every second day, of 30 cc. required for each experiment assisted in maintaining the anemia for this period. Immediately the blood was drawn it was placed in small bottles and shaken with glass beads until defibrinated. It was then filtered through a small wad of cotton wool to remove the clot and was then ready for use.

The blood was generally removed immediately before the experiment was set up. Occasionally, when kept for a few hours, it was placed in the cold room. Harrop (5) has shown that at low temperatures the viability of the cells is well maintained. In his experiments there was only a small reduction in the oxygen consumption of cells incubated at 38°C., after having been kept for 24 hours at 7°C., as compared with cells from the same blood which were incubated as soon as they were removed from the animal. My own observations are in agreement with his finding.

The oxygen consumption of the blood was determined in the respiration apparatus devised by Barcroft and modified by Warburg (6). 2 cc. of blood or of cell suspension were invariably used for the estimations. The oxygen consumption was determined at a temperature of 37°C.

The apparatus proved very satisfactory for this purpose; the oxygen consumption of this quantity of blood in flasks of about 15 cc. capacity usually causing a movement of the fluid meniscus in the manometer tube of some 10 to 20 cm. in the 90 minutes duration of the experiment. After the blood was placed in the apparatus the flasks were shaken about eighty times a minute for half an hour in the thermostat tank for the purpose of raising the temperature of the blood to 37°C. and also of fully oxygenating the red cells. The carbon dioxide given off was absorbed by normal sodium hydroxide placed in the small central cups in the flasks.

The possible sources of error in determining the oxygen consumption of blood have been discussed by Morawitz (7), by Warburg (3), and by Harrop (5) and

consequently need little consideration here. The duration of the experiments carried out by these investigators frequently extended over a period of many hours and consequently necessitated an aseptic technique. These observations were completed within 2 hours of the commencement of incubation and consequently no especial precautions were taken to ensure absolute sterility.

The question of the applicability of the Warburg method to this type of experiment requires more consideration. Two possible sources of error in the determinations need to be discussed. First, the evolution of carbon dioxide from the blood might possibly have some effect in causing the hemoglobin to take up small quantities of oxygen by a change in its affinity and so giving a spurious appearance of true oxidation by the red cells themselves. Secondly, all the hemoglobin may not be present in the form of oxyhemoglobin by the time the actual metabolism measurements are started. That no serious error from either of these causes occurs is shown by experiments upon the blood of normal rabbits containing only a small number of reticulocytes. Such blood, after the usual equilibration period of 30 minutes, shows a practically negligible oxygen consumption, indicating that by that time all the hemoglobin in the cells has become completely oxygenated. Further, this small consumption of oxygen continued with only slightly diminishing intensity for the ensuing 90 minutes, the duration of the experiment.

The degree of accuracy of the actual determinations may be judged from the following measurements of the oxygen consumption of the same samples of blood carried out simultaneously in six manometer tubes:

| Tube Numbers . . . . . | 1   | 2     | 3     | 4     | 5     | 6     |
|------------------------|---|-------|-------|-------|-------|-------|
|                        | C.mm oxygen consumed per cc. blood per hour |       |       |       |       |       |
| Expt. 1.....           | 123.8                                       | 122.2 | 122.4 | 122.0 | 124.0 | 123.6 |
| Expt. 2.....           | 64.0  | 63.5  | 64.6  | 63.6  | 64.3  | 63.5  |

## I

*Metabolism in Phenylhydrazine Anemia*

Phenylhydrazine has been extensively used in the production of experimental anemias in animals. It was first made use of in birds by Hirsch and Edel (8). Later it was employed by Kasarinoff (9) in his study of the primitive blood cells in fowls and his paper contains several excellent illustrations of the early red cell forms. When fowls are injected intraperitoneally with phenylhydrazine in medium doses, (20 mg. per kilo) in the course of a few minutes there is a striking



change in their appearance. Their combs assume a dusky brown color, and if blood be drawn at this stage it exhibits the same discoloration. This chocolate brown color of the blood persists for 48 to 72 hours before finally passing off, its termination being roughly coincident with the point of greatest severity in the anemia. The recovery from the anemia takes place fairly rapidly at first and then more slowly, being characterized by somewhat considerable oscillations in the red cell counts, a condition previously observed in the phenylhydrazine anemia of rabbits by Morawitz (7).

TABLE I

| Fowl No. | Days after injection | Red cell count in millions per c.mm. | Oxygen consumption           |                                      |                                 |
|----------|----------------------|--------------------------------------|------------------------------|--------------------------------------|---------------------------------|
|          |                      |                                      | C.mm. per cc. blood per hour | C.mm. per billion red cells per hour | Ratio of anemic to normal blood |
| 24       | 0                    | 3.27                                 | 29.7                         | 9.1                                  | —                               |
|          | 2                    | 1.12                                 | 118.2                        | 105.4                                | 11.6                            |
|          | 4                    | 1.98                                 | 163.8                        | 82.8                                 | 9.1                             |
|          | 6                    | 2.34                                 | 72.3                         | 30.9                                 | 3.4                             |
|          | 8                    | 1.76                                 | 29.8                         | 15.9                                 | 1.8                             |
|          | 10                   | 1.98                                 | 30.2                         | 15.2                                 | 1.7                             |
|          | 12                   | 1.80                                 | 31.1                         | 17.3                                 | 1.9                             |
| 25       | 0                    | 3.53                                 | 26.9                         | 7.6                                  | —                               |
|          | 2                    | 1.49                                 | 129.1                        | 86.7                                 | 11.4                            |
|          | 4                    | 2.27                                 | 164.2                        | 72.3                                 | 9.5                             |
|          | 6                    | 2.37                                 | 64.8                         | 27.3                                 | 3.6                             |
|          | 8                    | 2.27                                 | 37.3                         | 14.5                                 | 1.9                             |
|          | 10                   | 1.82                                 | 24.4                         | 13.4                                 | 1.7                             |
|          | 12                   | 2.23                                 | 37.7                         | 16.9                                 | 2.2                             |

Both fowls were injected with phenylhydrazine hydrochloride 0.5 per cent solution in saline (20 mg. per kilo) immediately after the blood was taken for the oxygen determinations on the first day of the experiment.

In this experiment observations were made both on the red cell counts and on the oxygen consumption of the blood. Two fowls were injected intraperitoneally with a one half per cent solution of phenylhydrazine in normal physiological saline (20 mg. per kilo). Immediately previous to the injection 12 cc. of blood were removed from each bird by cardiac puncture and its oxygen consumption determined. These latter were 9.1 and 7.6 c.mm. of oxygen per billion (one thousand

million) red cells per hour. Every second day afterwards 12 cc. of blood were similarly removed and the oxygen consumption and red cell count determined. The results are found in Table I and in Fig. 1.

The onset of the anemia was accompanied by a striking rise in the oxygen consumption of the cells, which at its peak attained a value of more than ten times its initial figure. At the same time the morpho-

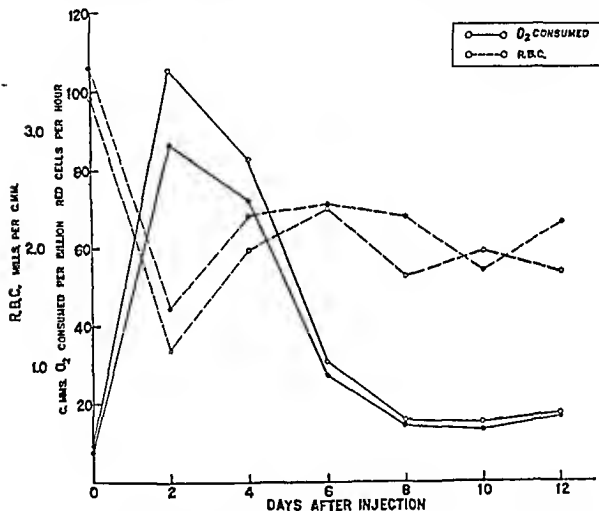


FIG. 1. Showing the oxygen consumption of avian red cells at various stages of an anemia produced by injection of phenylhydrazine.

logical appearances of the blood showed the marked changes described by Kasarinoff (9). Large numbers of the primitive hematoblasts were present together with many cells at all stages in the development of erythrocytes.

The enumeration of the various forms of primitive cells proved difficult on account of the many transitional varieties, and no attempt at division into specific categories was made other than into two large

groups: erythrocytes of normal appearance, and "primitive red cells" inclusive of all cells from the hematoblast to the large hemoglobin-containing cells still exhibiting vitally staining reticulum. Differential counts carried out in this way upon blood films, stained vitally with brilliant cresol blue followed by Wright's stain, showed that, at the peak of metabolism on the second day, in one blood 40 per cent and in the other 38 per cent, of the circulating cells fell into the category of "primitive red cells." With the decline in the intensity of the metabolism there was a corresponding fall in the proportion of these cells, development into the more advanced forms taking place in the blood stream. If the cells classified as normal merely possess the same oxygen consumption as similar cells present in the normal blood studied before the onset of the anemia, it is evident that the "primitive red cells" as a group have a metabolism some twenty to twenty-five times as great as the adult erythrocytes. Should the metabolism of normal blood be partly accounted for by the circulation of a small proportion of cells newly delivered from the bone marrow, and comparable with the reticulocytes of mammals, the difference between the oxygen consumptions of the two groups would be even greater.

The red cells which circulate in the blood of normal birds, though they retain their nuclei, are in an advanced state of degeneration so far as the normal cellular activities are concerned. Their respiration, which affords a measure of their metabolism, is low as judged by the respiration of other organs, while the process of pyknosis of their nuclei, which takes place in the later states of their development in the marrow, is indistinguishable from that taking place in the cells of other degenerating tissues. On the other hand the "primitive red cells" in the process of their transformation in the circulation into the normal adult erythrocytes consume oxygen at a rate of approximately 0.5 to 1.0 c.mm. per mg. per hour. While such an oxygen consumption is still somewhat smaller than that observed by Barcroft and Shore (10) in the cells of the liver of the cat, and by Usui (11) in those of the liver of the mouse, it must be recalled that the erythrocyte has included in its cytoplasm a relatively large quantity of oxyhemoglobin which has no oxygen consumption in the ordinarily accepted sense of that term. If allowance be made for this cellular inclusion the metab-

olism of the "primitive red cells" of the fowl becomes comparable with that of the cells of a parenchymatous organ like the liver. This relatively high metabolism, together with the resistance of the red cell to mechanical injury and its life in a fluid matrix of well determined composition, combines to render it a very suitable type of cell for the study of the influence of various factors upon cellular metabolism.

In the course of 8 days from the time of injection of the phenylhydrazine into the fowl the oxygen consumption of its blood had risen to a peak and returned nearly to its previous normal level. The persistent anemia was almost certainly due to the frequent removal of small quantities of blood for the purposes of the experiment. The continuous blood regeneration which just compensated for these losses was at the same time responsible for the somewhat elevated metabolism which was observed in the last few determinations made.

## II

### *Effect of Variation in Tonicity upon Metabolism*

The following experiments were carried out to determine how greatly metabolism was affected by variations in the tonicity of the saline solutions in which cells were suspended. The blood was obtained from fowls made anemic by an injection of phenylhydrazine. After centrifuging the blood for 5 minutes the supernatant serum was removed with a pipette and the cell suspension made up to its original volume with sodium chloride solutions of varying concentrations. Only one centrifugation was employed in order to minimize the inevitable injury to the cells caused by their being packed tightly together.

The observations are given in the following table:

| Red cells in millions per c mm. | NaCl: 0.6 per cent  | 0.75 per cent | 0.85 per cent | 0.95 per cent | 1.10 per cent |
|---------------------------------|---|---------------|---------------|---------------|---------------|
|                                 | C mm O <sub>2</sub> consumed per billion red cells per hour |               |               |               |               |
| 2 05                            | 32.2  | 32.0          | 32 0          | 33 2          | 32.8          |
| 1 33                            | 36.7  | 37.9          | 39 7          | 39.6          | 32.8          |
| 0 92                            | 41.7  | 42 6          | 44.8          | 44.6          | 41.1          |

While the effects of varying the tonicity of the saline solutions in which the cells are suspended are comparatively slight within the limits

employed in these experiments, it can be seen that the maximum metabolism occurs at some point between 0.85 per cent and 0.95 per cent of sodium chloride. Hematocrit observations made by the method described by Hirota (12) demonstrated that between these concentrations the size of the suspended red cells both in normal and anemic blood approximates most closely to that when they are present in serum.

| Red cells in millions per c.mm. | Serum   | 0.75 per cent | 0.85 per cent | 0.90 per cent | 0.95 per cent | 1.05 per cent |
|---------------------------------|---|---------------|---------------|---------------|---------------|---------------|
|                                 | Hematocrit readings in per cent in NaCl solutions |               |               |               |               |               |
| 2.76                            | 28.5  | 31.3          | 29.3          |               | 27.6          | 27.2          |
| 1.36                            | 24.3  |               |               | 24.2          |               |               |

These results are similar to those obtained by Ray (13) for the reticulocytes of the dog. He made observations on the oxygen consumption of these cells in the presence of  $M/12.5$  solutions of sodium lactate to which sufficient sodium chloride was added to render them isotonic with the following concentrations of sodium chloride: 0.5, 0.7, 0.9, 1.1, 1.3 per cent. He found that the oxygen consumption attained its maximum in solutions isotonic with 0.9 per cent sodium chloride and that it declined rather more rapidly if the solutions were made hypertonic than if they were made hypotonic.

### III

#### *The Influence of Hydrogen Ion Activity upon Metabolism*

The extent to which the hydrogen ion activity of the surrounding medium influenced metabolism in the avian red cell was determined in this series of experiments. The method employed here differed somewhat from that adopted in the other studies. The oxygen consumption of the cells was measured in their own serum to which certain known quantities of acid had been added. In order to examine whether any specific ionic effects were involved both hydrochloric and phosphoric acids were used.

To 2 cc. of blood from an anemic fowl 1 cc. of 0.87 per cent sodium chloride solution containing known amounts of these acids was added. The acidity of these saline solutions varied between  $N/200$

TABLE II

*The Effect of Variations in Hydrogen Ion Activity upon the Metabolism of Avian Red Cells*

| Concentrated (mols per l.)<br>HCl in 0.87 per cent NaCl<br>solution added to cells | C.mm.<br>O <sub>2</sub> | Per cent<br>of max.<br>O <sub>2</sub> con-<br>sump-<br>tion | pH | C.mm.<br>O <sub>2</sub> | Per cent<br>of max.<br>O <sub>2</sub> con-<br>sump-<br>tion | pH   | C.mm.<br>O <sub>2</sub> | Per cent<br>of max.<br>O <sub>2</sub> con-<br>sump-<br>tion | pH   |
|--|-------------------------|---|----|-------------------------|---|------|-------------------------|---|------|
| C.   |                         |   |    |                         |   |      |                         |   |      |
| 0.000  | 45.0                    | 86  | —  | 34.1                    | 85  | 8.65 | 33.6                    | 87  | 8.79 |
| 0.005  | 45.6                    | 88  | —  | 36.1                    | 90  | 8.53 |                         |   |      |
| 0.010  | 48.9                    | 94  | —  | 37.6                    | 94  | 8.03 | 36.4                    | 94  | 8.02 |
| 0.020  | 52.1                    | 100   | —  | 40.0                    | 100   | 7.80 | 38.7                    | 100   | 7.90 |
| 0.030  |                         |   |    |                         |   |      | 38.8                    | 100   | 7.64 |
| 0.040  |                         |   |    |                         |   |      | 35.2                    | 91  | 7.44 |
| 0.050  | 45.3                    | 87  | —  | 35.2                    | 88  | 7.27 | 28.7                    | 74  | 7.22 |
| 0.100  | 27.1                    | 52  | —  | 19.0                    | 48  | 5.85 |                         |   |      |
| Red cell counts in<br>millions.....  | 1.75                    |   |    | 1.96                    |   |      | 2.15                    |   |      |

| Concentrated (mols per l.)<br>H <sub>2</sub> PO <sub>4</sub> in 0.87 per cent NaCl<br>solutions added to cells | C.mm.<br>O <sub>2</sub> | Per cent<br>of max.<br>O <sub>2</sub> con-<br>sump-<br>tion | pH   | C.mm.<br>O <sub>2</sub> | Per cent<br>of max.<br>O <sub>2</sub> con-<br>sump-<br>tion | pH   | C.mm.<br>O <sub>2</sub> | Per cent<br>of max.<br>O <sub>2</sub> con-<br>sump-<br>tion | pH   |
|--|-------------------------|---|------|-------------------------|---|------|-------------------------|---|------|
| C.   |                         |   |      |                         |   |      |                         |   |      |
| 0.000  | 29.6                    | 92  | 8.34 | 47.4                    | 91  | 8.26 | 45.0                    | 84  | 8.46 |
| 0.005  |                         |   |      |                         |   |      | 45.9                    | 86  | 8.19 |
| 0.010  | 30.8                    | 96  | 8.04 | 51.9                    | 100   | 8.10 | 49.8                    | 93  | 8.08 |
| 0.015  |                         |   |      |                         |   |      | 53.4                    | 100   | 7.88 |
| 0.020  | 32.1                    | 100   | 7.65 | 52.0                    | 100   | 7.53 | 53.5                    | 100   | 7.62 |
| 0.025  |                         |   |      |                         |   |      | 45.1                    | 84  | 7.30 |
| 0.030  | 24.9                    | 78  | 7.28 | 40.3                    | 77  | 7.18 |                         |   |      |
| 0.040  | 21.0                    | 65  | 6.91 | 32.3                    | 62  | 6.79 |                         |   |      |
| 0.050  | 18.6                    | 58  | 6.66 | 29.8                    | 57  | 6.56 |                         |   |      |
| Red cell counts in<br>millions.....  | 2.12                    |   |      | 1.44                    |   |      | 1.18                    |   |      |

\* Oxygen consumption given in units of c.mm. O<sub>2</sub> per billion red cells per hour.

and N/10. 2 cc. of this cell suspension were used for the determination of its oxygen consumption. Immediately after the measurement of the metabolism was completed the suspension of cells was removed from the flasks of the Barcroft-Warburg manometers, rapidly centri-

fuged and the supernatant fluid removed. This separation of the cells was effected as quickly as possible to minimize any change in reaction of the solutions by the further metabolism of the red cells.

The reaction of the supernatant fluid was measured with the quinhydrone electrode. I am indebted to Dr. Arda Alden Green for checking a number of the more important points on the hydrogen

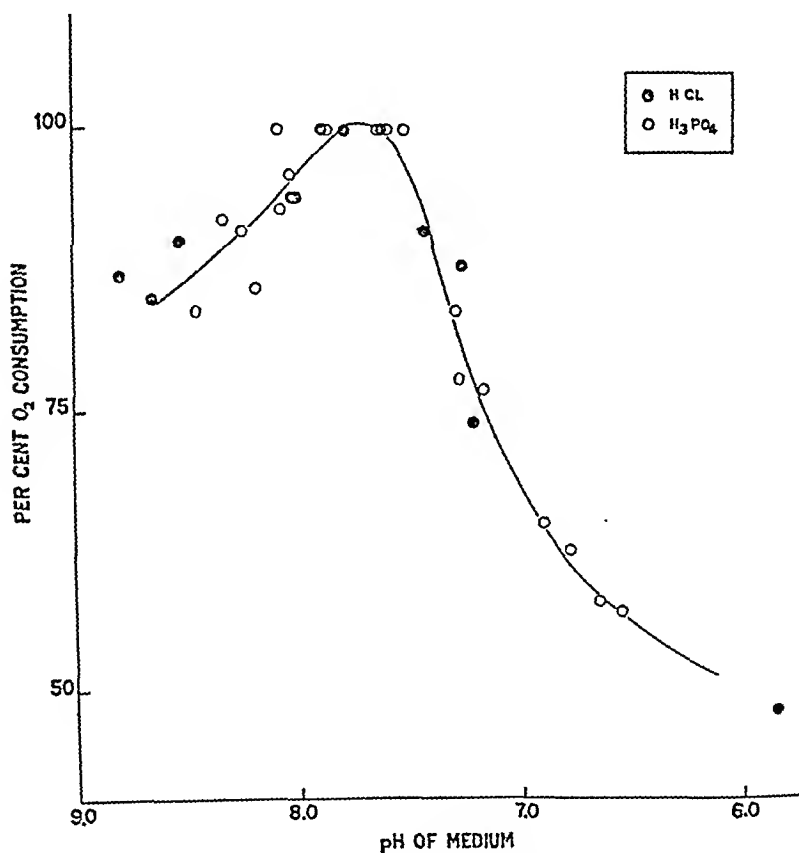


FIG. 2. Showing the influence of changes in hydrogen ion activity upon the oxygen consumption of primitive avian red cells.

electrode. The agreement between the two methods proved satisfactory up to a reaction of about pH 8.4.

The relative alkalinity of the sera, to which saline only was added, was due to the loss of a large part of its carbon dioxide in the first few minutes of shaking in the apparatus. This carbon dioxide was absorbed in the sodium hydroxide which was placed in the central cups

of the flask in order to combine with the carbon dioxide given off by the cells in the course of their metabolism.

The results are presented in Table II and in Fig. 2.

These experiments demonstrate that the addition of small quantities of acid to the serum in which the cells are suspended exercises a very considerable influence upon the oxygen consumption of the red cells themselves. This effect appears to be dependant upon the hydrogen ion concentration of the resulting mixture, it being immaterial whether this reaction be reached by the addition of phosphoric acid or hydrochloric acid. In the case of certain other acids their presence in concentrations necessary to bring about the range of reaction investigated here would most probably introduce specific ionic effects other than that from the dissociated hydrogen ion.

The range of variation studied was considerable: the lowest point being pH 5.85, and the highest pH 8.79. The maximum oxygen consumption took place at pH 7.75. In solutions of this reaction the contents of the corpuscles would probably have a considerably more acid reaction. Taylor (14) has estimated that at this reaction the corpuscular contents in goose blood have a pH value nearly 0.3 lower than the serum. On either side of this reaction the metabolism rapidly declined, until on the acid side at a pH of 6.5 it was only half of its value at the maximum. On the alkaline side the falling away did not seem to be so steep, but since the quinhydrone electrode is inaccurate at reactions more alkaline than pH 8.4 the figures in this range are less dependable. It is of interest that Fiscber (15) in his investigations of the influence of hydrogen ion activity upon the growth and emigration of fibroblasts in tissue cultures found that these took place at their best between pH 7.4 and pH 7.8. He also observed that repeated cultivation in a medium on the alkaline side of pH 7.4 was much less injurious to the cells than cultivation in one more acid.

There exists the possibility that the hydrogen ion concentration of the media might undergo change during the course of the experiment and that the final determinations might give a false indication of the reaction at which the maximum oxygen consumption took place. Should the reaction change appreciably within the 90 minutes of the experiment it might be expected that, since the red cells are evidently



quite sensitive to such a change, the maximal metabolism in the last 45 minutes would be observed in a different observation flask from that of the experiment as a whole. This did not take place. In every case the flask of blood which registered the highest oxygen consumption for the whole experiment also registered the highest if the last 45 minutes were alone considered. It is consequently highly improbable that the reactions in the individual flasks varied appreciably in the period of observations.

#### IV

##### *Effect of Variations of Glucose Concentrations upon Metabolism*

A series of observations was made to determine the effect of the presence of small quantities of glucose upon the oxygen consumption of the red blood cells in the blood of anemic birds. The technical procedures were similar to those employed in the studies of the influence of tonicity. The blood from anemic fowls was defibrinated by shaking with glass beads and centrifuged for a period of 5 minutes in a series of graduated tubes. After the supernatant serum was removed the cell suspensions were restored to their original volumes by the addition of sodium chloride solutions (0.87 per cent) containing varying quantities of glucose. In the last experiment, where the glucose was added in rather high concentration, that of the sodium chloride was correspondingly reduced. It was found, by hematocrit determinations, that the cells both of normal and anemic fowls' blood had the same volume in 4.8 per cent glucose solutions as they had in their own serum and the concentrations of the sodium chloride present in the last experiment were adjusted upon this basis. No adjustment was made for variations in tonicity in the earlier experiments where lower concentrations of glucose were used. Previous observations have however shown that greater alterations in tonicity than were involved here have little influence upon the oxygen consumption of the cells, and this influence would in any case have been in the direction of reduction rather than acceleration of metabolism.

Table III and Fig. 3 show the results obtained.

It is evident that small increments in the concentration of the glucose present are accompanied by corresponding acceleration in the oxygen

consumption of the cells. With further increase in the amount of glucose, however, the metabolism was stimulated less and less until from 0.6 per cent to 1.2 per cent no change in the consumption of oxygen took place. The metabolism between these two points was steady at a level some 15 per cent above that in the corresponding glucose-free saline solution. We have not determined how far this condition of steady oxygen consumption continues with rising glucose concentration. Warburg (3) states that the blood cells in anemic geese consume the same quantity of oxygen in a 5 per cent glucose

TABLE III

*C.mm. O<sub>2</sub> Consumed per Billion Red Cells Per Hour in Varying Concentrations of Glucose*

| Red cells in millions<br>per c mm . . . . . | 0.91                 |                                | 1.66                 |                                | 1.93                |                                | 1.98                |                                |
|---|----------------------|--------------------------------|----------------------|--------------------------------|---------------------|--------------------------------|---------------------|--------------------------------|
| Concentration<br>glucose                    | C mm. O <sub>2</sub> | Per cent<br>of saline<br>value | C.mm. O <sub>2</sub> | Per cent<br>of saline<br>value | C mm O <sub>2</sub> | Per cent<br>of saline<br>value | C.mm O <sub>2</sub> | Per cent<br>of saline<br>value |
| <i>per cent</i>                             |                      |                                |                      |                                |                     |                                |                     |                                |
| nil   | 77.1                 | 100                            | 122.9                | 100                            | 96.0                | 100                            | 72.4                | 100                            |
| 0.1   | 81.4                 | 105                            | 130.4                | 106                            | 102.0               | 106                            |                     |                                |
| 0.2   | 83.6                 | 108                            | 132.0                | 107                            | 102.0               | 106                            |                     |                                |
| 0.3   | 84.8                 | 110                            | 132.5                | 108                            | 104.5               | 109                            | 81.6                | 113                            |
| 0.4   |                      |                                | 133.8                | 109                            |                     |                                |                     |                                |
| 0.5   |                      |                                | 134.3                | 110                            | 106.5               | 111                            |                     |                                |
| 0.6   |                      |                                |                      |                                |                     |                                | 83.6                | 115                            |
| 0.75  |                      |                                |                      |                                | 109.6               | 114                            |                     |                                |
| 0.9   |                      |                                |                      |                                |                     |                                | 83.4                | 115                            |
| 1.2   |                      |                                |                      |                                |                     |                                | 83.4                | 115                            |

solution as they do in Ringer's solution, which would imply a decline beyond a maximal plateau. I have previously carried out determinations (16) of the sugar concentration present in the serum of normal starved fowls and found it to be from 0.20 to 0.22 per cent. It is clear therefore that the maximal oxygen consumption of the red cells takes place at a glucose concentration several times that normally present in the serum.

The permeability of erythrocytes to glucose has been extensively studied, but so far as I have been able to find, in every case upon the normal fully developed cells and in nearly all cases upon those of

mammals. However since the metabolism measured in this study is almost exclusively that of the "primitive red cells" these observations upon permeability do not necessarily apply to them. It would seem improbable that the oxygen consumption of cells could be increased unless the glucose actually entered the cells. A control experiment showed that no interaction, involving the consumption of oxygen,

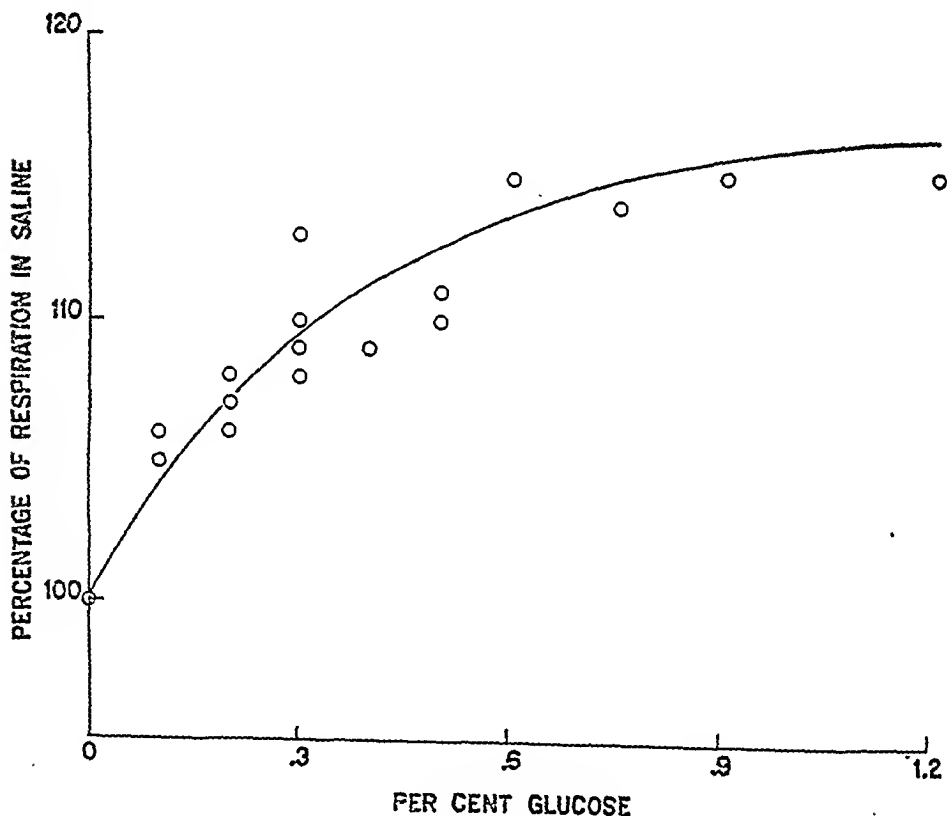


FIG. 3. Showing the influence of glucose concentration upon the oxygen consumption of primitive avian red cells.

took place when small quantities of cell-free serum were added to the glucose solutions used.

A further consideration indicating that the cells are permeable to glucose is provided by observations on the gradual decline in the oxygen consumption of the red cells as the duration of the experiment is extended. Ordinarily the period over which the observations were made was 2 hours and even in the course of that time a gradual de-

crease in the rate of consumption of oxygen could be observed. If this period were much extended the decline would become quite obvious. This decrease however was much less rapid in those solutions containing much glucose than in those with none. It would consequently seem that the oxidative processes involve the gradual depletion of some constituent of the cell which the presence of glucose in the surrounding medium could in some degree replace. This confirms the results of Grafe (17) who found that in Locke's solution containing glucose the cells respired almost as vigorously in a second experimental period of 90 minutes as they did in the first one of the same length of time.

The nature of the substances oxidized in the red cell is not known. Harrop and Barron (18) have carried out a few experiments upon the respiration of avian (goose) red cells, both in normal and anemic blood, in which they determined both the oxygen consumption and the carbon dioxide production. The resulting respiratory quotients were however too variable to give any indication of the character of the materials consumed. That these materials are, however, mainly within the cells themselves is shown by the fact that the oxygen consumption of cells suspended in an isotonic saline solution is not greatly inferior to that of cells from the same source in their own serum.

## V

### *The Influence of Certain Amino Acids on Metabolism*

Studies upon the metabolism of man and animals have shown that proteins and products of protein hydrolysis possess an especially great specific dynamic action. The following group of experiments was carried out to determine whether this stimulation of metabolism by amino acids could be reproduced on avian red blood cells *in vitro*.

In these experiments, as in those in which glucose was studied, the serum was separated from the cells by centrifugalization and replaced by solutions of various amino acids in 0.87 per cent sodium chloride. The amino acid solutions were neutralized with sodium hydroxide to pH 7.6. The buffering power of such solutions is very slight compared to that of the cells to which they were added. In each experiment the amino acid solutions added to the cells varied in concentration from

zero upwards to 50 mg. amino acid nitrogen per 100 cc. In a previous series of determinations I have found that serum of normal starving fowls contains from 14 to 18 mg. amino acid nitrogen per 100 cc. as estimated by the colorimetric method of Folin and Wu. Consequently the maximum concentration of amino acid used was approximately three times as great as in the serum of the starving fowl.

TABLE IV

*Effect of Amino Acids upon the Oxygen Consumption of Red Cells*

| Amino acid              | Millions<br>per c.mm. | Concentration of amino acid (mg. amino N per cent)           |               |               |               |               |               |              |              |
|-------------------------|-----------------------|--|---------------|---------------|---------------|---------------|---------------|--------------|--------------|
|                         |                       | nil.   | 1 mg.         | 2 mg.         | 3 mg.         | 5 mg.         | 10 mg.        | 25 mg.       | 50 mg.       |
|                         |                       | C.mm. O <sub>2</sub> consumed per billion red cells per hour |               |               |               |               |               |              |              |
| Glycine                 | 1.82                  | 34.7<br>(100)  |               |               |               | 33.5<br>(97)  | 33.5<br>(97)  | 30.2<br>(87) | 29.2<br>(84) |
|                         | 1.39                  | 92.8<br>(100)  |               |               |               | 90.9<br>(98)  | 85.3<br>(92)  | 72.0<br>(78) | 66.1<br>(71) |
|                         | 1.67                  | 34.7<br>(100)  | 34.8<br>(100) | 34.7<br>(100) | 34.2<br>(99)  | 33.3<br>(96)  |               |              |              |
| Alanine                 | 1.47                  | 44.3<br>(100)  | 44.8<br>(101) | 45.9<br>(103) | 44.7<br>(101) | 44.8<br>(101) | 44.5<br>(100) |              |              |
| Histidine               | 1.58                  | 48.1<br>(100)  |               |               |               | 47.0<br>(98)  | 47.1<br>(98)  | 46.4<br>(97) |              |
| <i>l</i> -aspartic acid | 1.17                  | 55.3<br>(100)  |               |               |               | 55.2<br>(100) | 55.5<br>(100) |              |              |

The figures in brackets represent the oxygen consumption in percentage of that taking place in the saline medium.

The actions of four amino acids were investigated: glycine, alpha alanine, histidine and *l*-aspartic acid. The latter was studied because Burge, Wickwire, Estes, and Williams (19) had found that certain optically active amino acids, amongst them *l*-aspartic acid, exerted a stimulating effect upon the metabolism of *Paramecium*, which was not present in the optically inactive ones. Glycine and alanine on the other hand are of interest since Lusk (20) has shown them to have

especially high specific dynamic actions when tested on the metabolism of dogs.

The results are shown in Table IV.

It is clearly evident from these experiments that none of the amino acid solutions employed caused any elevation of the oxygen consumption of the fowls' red cells in any way comparable to their specific dynamic action in the intact animal. At low concentrations their effect is negligible; alanine being the only one to produce any increment and that of an order little greater than the experimental errors of the determinations. At higher concentrations than 5 mg. of amino acid nitrogen per 100 cc., glycine caused a very definite and increasing reduction in the oxygen consumption of the cells. The other three amino acids, alanine, histidine, and *L*-aspartic acid were without effect in concentrations up to 10 mg. of amino acid nitrogen per 100 cc., a concentration which it is unlikely that they ever exceed in the serum of the intact animal even at the height of digestion.

The particular component of the amino acids responsible for the specific dynamic action has long been a subject of controversy. Grafe (17) (21) has contributed to the problem from two aspects. Working originally with Warburg and employing his methods, he made a study of the action of ammonia and of a number of amines upon the oxygen consumption of the red cells of the blood of anemic geese. He observed that if 10 cc. of a neutralized saline solution containing  $1/100$  N  $\text{NH}_4\text{Cl}$  were added to 2 cc. of a concentrated suspension of red cells, their metabolism became approximately 60 per cent greater than those in a similar cell suspension to which saline only had been added. If, however, the cells were washed three times with this ammonium chloride solution before the oxygen consumption determinations were made the metabolism of the cells fell to approximately one-fifth of those in saline. This he accounted for by the specific absorption of ammonia by the cells, each washing contributing an additional quota to the cell contents. Consequently after three washings the cells contained considerably more ammonia than they would have absorbed from a single immersion in a solution of the same concentration. Provided the concentration of ammonia were not raised to a point at which hemolysis occurred, the inhibition of metabolism was reversible, for washing the cells from the ammonia

solutions in normal physiological saline restored the oxygen consumption to its original level. Apparently immersion in a saline solution containing 1/100 N  $\text{NH}_4\text{Cl}$  (14 mg. ammonia nitrogen per cent) raised the metabolism very considerably while the higher concentrations consequent upon repeated washings in the same solution resulted in a very considerable though reversible inhibition of the oxygen consumption.

Grafe's second contribution to this question was his study of the relative specific dynamic actions of various amino acids and ammonium chloride upon both men and animals. From the combination of his own observations with those of Lusk, Grafe comes to the conclusion that the specific dynamic action of the various amino acids is closely related to their nitrogen content. This, taken in conjuncture with the observation that, though containing no calorie content available to the body, ammonium chloride produces a pronounced stimulation of metabolism in man, makes him suggest that all these substances produce their action by reason of the amino groups which they contain. His experiments with ammonium chloride on rabbits and dogs were inconclusive.

I have carried out certain observations upon the effects of ammonium chloride at various concentrations upon the oxygen consumption of the red cells in the blood of anemic fowls. The solutions of ammonium chloride were made up and neutralized to a pH of 7.6 in exactly the same way as for the amino acids. The solutions in various concentrations were added to the concentrated cell suspensions to replace the serum removed after centrifugalization. In this way they resemble the experiment of Grafe in which he observed a very considerable stimulation of metabolism. The results are to be found in Table V.

The results of these experiments are somewhat equivocal. In all five the effect of the ammonium chloride was to produce a rise in the oxygen consumption of the cells. In two fowls, one examined twice, this stimulation was slight and attained a maximum at concentrations of between 1 and 2 mg. of nitrogen per 100 cc. In higher concentrations the metabolism of these bloods showed a progressive decline. Even at the maximum the elevation of metabolism was slight, being only approximately 5 per cent. In one fowl, also examined twice,

increase in the concentration of the ammonium chloride produced a rise in the oxygen consumption that continued as far as the maximum concentration used in experiments. The cause of the divergency in the reaction of the red cells to the ammonium chloride solutions is not evident and has not been further investigated. One difference between the blood of this animal and the others was the low oxygen

TABLE V

*Effect of Ammonium Chloride upon the Oxygen Consumption of Red Cells*

| Fowl Number | Red blood corpuscles.<br>Millions per c.mm. | Concentration of ammonium chloride (mg. N per cent)          |               |               |               |               |               |
|-------------|---|--|---------------|---------------|---------------|---------------|---------------|
|             |   | nil.   | 1 mg.         | 2 mg.         | 5 mg.         | 10 mg.        | 25 mg.        |
|             |   | C.mm. O <sub>2</sub> consumed per billion red cells per hour |               |               |               |               |               |
| 2           | 2.36  | 17.8<br>(100)  | 19.0<br>(107) | 20.1<br>(113) | 20.5<br>(115) | 20.1<br>(113) | 21.1<br>(119) |
| 4           | 1.54  | 37.0<br>(100)  | 38.1<br>(103) | 38.8<br>(105) | 36.3<br>(98)  | 35.1<br>(95)  | 34.7<br>(94)  |
| 5           | 1.91  | 35.4<br>(100)  | 37.2<br>(105) | 37.2<br>(105) | 35.6<br>(100) | 33.2<br>(94)  | 33.0<br>(93)  |
| 2           | 1.98  | 15.7<br>(100)  | 17.2<br>(109) | 17.9<br>(114) | 18.6<br>(118) | 18.9<br>(120) | 19.4<br>(123) |
| 5           | 1.74  | 47.4<br>(100)  | 49.0<br>(105) | 48.6<br>(104) | 46.3<br>(98)  | 42.3<br>(89)  | 40.7<br>(86)  |

The figures in brackets represent the oxygen consumption in percentage of that taking place in the saline medium.

consumption per billion red cells per hour, a figure only about twice that of the blood of normal fowls.

Grafe (17) in his experiment used a solution of ammonium chloride to which a molecular equivalent of sodium hydroxide had been added, so releasing free ammonia in the solution. The concentration of this ammonia was 14 mg. of ammonia nitrogen per 100 cc. Such a solution must have had an alkaline reaction at the time of its addition to the cells, though the presence of carbon dioxide in the cell suspension would ensure its rapid conversion into ammonium carbonate. In



his experiment with the red blood cells of anemic geese he observed a rise in oxygen consumption of 60 per cent. Such an elevation is far greater than any observed in the bloods of the fowls examined here. At concentrations of ammonia nitrogen comparable to his, two of the fowls' bloods showed a decrease of about 7 per cent, while the other showed an elevation of about 17 per cent.

### CONCLUSIONS

1. The oxygen consumption of normal and "primitive red cells" of fowls' blood has been determined at intervals in the course of an anemia produced by the injection of phenylhydrazine. The "primitive red cells" have an oxygen consumption at least twenty to twenty-five times greater than the normal red cells.

2. Suspension of the cells derived from the blood in anemia in sodium chloride solutions of various concentrations has comparatively little effect upon the oxygen consumption of the cells.

3. The red cells from anemic blood are sensitive to variations in the reaction of the medium in which they are suspended. The maximum oxygen consumption, after addition of a saline solution containing variable amounts of acid to the blood, took place at pH 7.75. They appeared somewhat more sensitive to variations on the acid side of this reaction than on the alkaline.

4. Addition of glucose to the medium increased the oxygen consumption of the cells. Their metabolism in a physiological saline solution containing 0.6 per cent of glucose was 15 per cent higher than in one in which no glucose was present.

5. Certain amino acids in low concentrations had little effect on oxygen consumption, though at higher concentrations some of them definitely diminished it.

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# FACTORS INFLUENCING THE RESPIRATION OF ERYTHROCYTES

## II. MAMMALIAN RETICULOCYTES

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Cohnstein and Zuntz (1) were the earliest investigators who clearly pointed out that certain types of blood exhibit a metabolism involving the disappearance of oxygen and reduction of the hemoglobin. This metabolism was most evident in fetal blood, which, they observed, gradually darkened in color upon standing and upon evacuation liberated less oxygen than before. They ascribed this loss of oxygen to the action of the red cells, many of which, at the stage of development of the blood which they examined, still possessed large vesicular nuclei.

Since then the metabolism of the blood has been very actively studied, notably by Warburg, by Morawitz, and by Harrop. The rather extensive literature has been well reviewed by Morawitz (2). In general, it may be summarized by stating that it is now agreed that the normal orthochromatic erythrocytes, which form the vast majority of the circulating red cells of mammals, have a negligible if any respiration (Daland and Isaacs (3)). On the other hand the polychromatic cells, which have been recently liberated from the bone marrow and which are found in large numbers in young animals and during active blood regeneration, exhibit an easily measurable consumption of oxygen and formation of carbon dioxide. The earlier nucleated cells, ordinarily present only in the bone marrow in mammals, and the nucleated circulating erythrocytes of birds and amphibia, also respire very actively.

With the exception of Warburg's studies (4), which were largely concerned with the manner in which respiration was inhibited by

various narcotics, the factors which influence this metabolism have not been extensively investigated. This paper deals with the variations in the respiration of the reticulocytes of the rabbit brought about by various alterations in the medium in which they are suspended.

### *Methods*

The majority of the methods used in this investigation have already been described in the previous paper. The blood used was obtained from the ear vein and was defibrinated as it was collected by shaking with glass beads. Before use it was filtered through cotton wool to remove the coagulated fibrin and with it the large majority of leucocytes (Warburg (5)).

The rabbits were made anemic by intraperitoneal injections of phenylhydrazine hydrochloride (15 mg. per kilo) dissolved in normal physiological saline solution. The anemia reached its greatest severity in about 7 days. The blood used in these experiments was removed from animals at a time when there was a large proportion of reticulocytes and consequently a high rate of respiration.

### *The Respiration of the Blood during the Onset and Recovery Stages in Phenylhydrazine Anemia*

An injection of phenylhydrazine into a rabbit is rapidly followed by the development of anemia. With suitable quantities of the substance (15 to 20 mg. per kilo) the red cell count may be caused to fall from its normal value of rather over five million to between one and two million red cells per c. mm. Regeneration, however, sets in very quickly, and by the time that the anemia has become most severe, between the sixth and eighth days, the blood may contain enormous numbers of reticulocytes. When the red cell count falls below two million per c. mm., more than 50 per cent of the cells may be reticulocytes, or more than one million per c. mm. More commonly the number lies between six and eight hundred thousand per c. mm. If the oxygen consumption of such a specimen of blood be determined it will be found to have a very much higher value than that from a normal rabbit. Harrop (6) has shown that a rough proportionality exists between the number of reticulocytes in, and the oxygen consumption of, various pathological human bloods. Similarly Derra (7) studied the oxygen consumptions of blood during the remissions resulting from the administration of liver extract in patients suffering from pernicious anemia. He found a similar relationship, though not so close as that

observed by Harrop, between the oxygen consumption and the reticulocyte content of the blood at various times in the remissions.

In the following experiment two rabbits were made anemic by injection with phenylhydrazine. Red cell counts, reticulocyte counts and oxygen consumption determinations were made every second day until the main wave of liberation of reticulocytes had subsided. The observations are to be found in Table I and Fig. 1.

TABLE I

*The Oxygen Consumption of Reticulocytes at Various Phases in the Anemia Induced by the Injection of Phenylhydrazine*

| Rabbit No. | Day               | Red cells in mills per c mm. | Retics per cent | Retics. in mills per c.mm | C mm. O <sub>2</sub> consumed |                              |
|------------|-------------------|------------------------------|-----------------|---------------------------|-------------------------------|------------------------------|
|            |                   |                              |                 |                           | Per cc. blood per hour        | Per billion retics. per hour |
| 28         | before inject.    | 5.49                         | 1.5             | 0.083                     | 5.1                           | 63.0                         |
|            | 1st after inject. | 6.01                         | 3.2             | 0.192                     | 11.1                          | 57.8                         |
|            | 3rd " "           | 2.85                         | 9.3             | 0.265                     | 17.0                          | 64.2                         |
|            | 5th " "           | 2.40                         | 27.0            | 0.649                     | 41.9                          | 64.7                         |
|            | 7th " "           | 2.44                         | 33.0            | 0.805                     | 52.9                          | 65.5                         |
|            | 9th " "           | 3.80                         | 17.2            | 0.654                     | 35.5                          | 54.3                         |
|            | 11th " "          | 4.07                         | 10.2            | 0.423                     | 25.4                          | 59.9                         |
|            | 13th " "          | 4.90                         | 9.6             | 0.470                     | 26.0                          | 55.3                         |
| 40         | before inject.    | 4.97                         | 2.0             | 0.099                     | 5.3                           | 53.6                         |
|            | 1st after inject. | 4.96                         | 3.0             | 0.149                     | 8.9                           | 59.7                         |
|            | 3rd " "           | 3.32                         | 9.4             | 0.313                     | 14.9                          | 47.6                         |
|            | 5th " "           | 2.64                         | 23.9            | 0.631                     | 40.0                          | 63.2                         |
|            | 7th " "           | 3.15                         | 21.0            | 0.630                     | 35.2                          | 55.9                         |
|            | 9th " "           | 3.67                         | 13.1            | 0.479                     | 25.3                          | 52.8                         |
|            | 11th " "          | 3.19                         | 9.1             | 0.290                     | 15.2                          | 52.4                         |
|            | 13th " "          | 3.61                         | 5.3             | 0.192                     | 10.9                          | 56.7                         |

From Table I it can be seen that the actual number of reticulocytes per c.mm. of blood increased, in one animal to more than nine, in the other to more than six, times their initial figures, and that the oxygen consumption of the blood underwent a concurrent change. But in spite of the very large alteration in both, the oxygen consumption for every billion (thousand million) reticulocytes was, within reasonably close limits, the same throughout the course of the experiment.

There are at least two possible explanations for this rather strikingly uniform relationship between oxygen consumption and reticulocyte concentration. In the first place it is possible that the reticulum in the cell either promotes oxidation or is itself oxidized at the same rate at all stages in its gradual disappearance. When taken in conjunction with the cytological appearances of the reticulocytes with their very variable content of basophil filaments, such an assumption seems

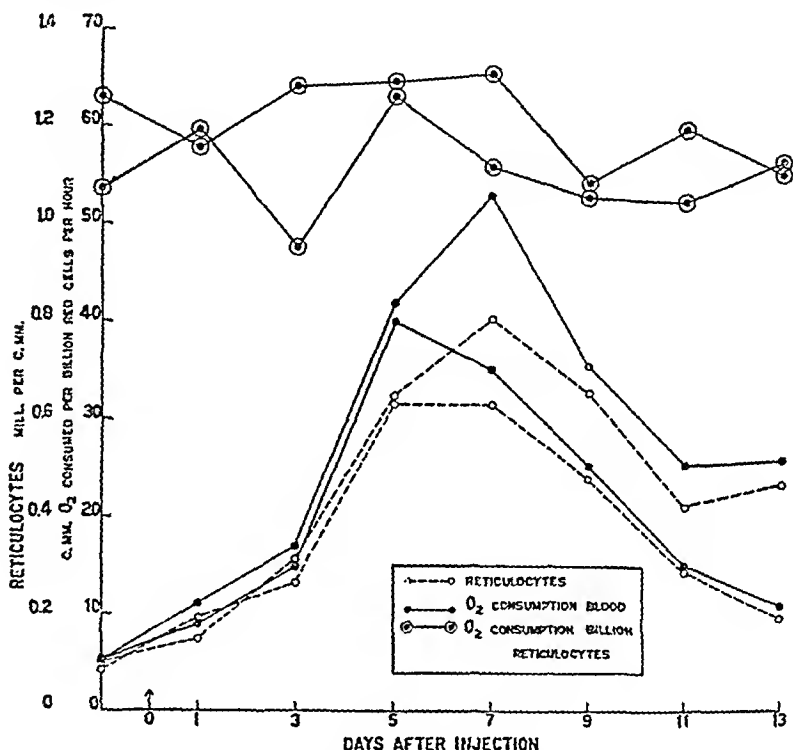


FIG. 1. Showing the oxygen consumption of reticulocytes related to their concentration in the blood at various stages of an anemia produced by phenylhydrazine.

hardly probable. A second and seemingly more likely explanation is that the reticulocyte stage of development is of comparatively short duration in comparison with the whole duration of the wave of reticulocyte production evoked by the phenylhydrazine. Under these circumstances the oxygen consumption curve for the blood would represent, at any one particular time, the summation of the oxygen consumption of reticulocytes at all stages in their life history. In this

event, though the total number of reticulocytes in the circulation might change greatly, the proportions of the various categories of cells, young and old, might not be very different at different times.

An accurate estimation of the duration of the reticulocyte stage of development would determine this point. Subject to some assumptions which will be discussed below, this period can be indirectly estimated from a consideration of the regeneration curves for the blood of rabbits recovering from anemia. In the course of this recovery the red cell count of the animal rises from the day of most severe anemia until regeneration is complete. If daily total red cell counts and reticulocyte percentage counts be made, the actual number of reticulocytes per c. mm. of blood can be calculated for any particular day. Should, on an average, each reticulocyte retain its reticulum for only 1 day before developing into an ordinary orthochromatic erythrocyte, it will, taken in the aggregate, be only counted once as a reticulocyte in the daily blood examinations made. Consequently, if the life of the reticulum once the cell has reached the circulating blood be only 1 day, the total daily calculated numbers of reticulocytes added together for the whole regeneration period should be equal to the rise in the red cell count during the same period. If the aggregate number of the reticulocytes so counted be greater than the number of new cells produced, as judged by the rise in the total red cell count, the average duration of the reticulum in the cells must be longer than 1 day, since they must have been counted more than once.

Such a calculation obviously makes several important assumptions. First, it presupposes that all the red cells added to the circulation in the period of regeneration are liberated from the bone marrow as reticulocytes and not as the normal adult orthochromatic variety of red cell. It is impossible at the present time to be certain whether this is so or not, since there are no satisfactory ways of recognizing new cells other than those of vital staining. If considerable numbers of non-reticulated red cells are liberated the calculation just outlined will estimate the duration of the reticular stage of development at too brief a period. It further disregards any alteration in blood volume and the normal or abnormal destruction of red cells, either adult or reticulated, during the regeneration period. How far these might affect the result it is clearly impossible to estimate.



TABLE III

*The Influence of Hydrogen Ion Activity upon the Oxygen Consumption of Reticulocytes*

| Concentration of acid or alkali added | C.mm. O <sub>2</sub> per bill. retics. hourly | Per cent of value in serum saline | pH   | C.mm. O <sub>2</sub> per bill. retics. hourly | Per cent of value in serum saline | pH   | C.mm. O <sub>2</sub> per bill. retics. hourly | Per cent of value in serum saline | pH   |
|---------------------------------------|---|-----------------------------------|------|---|-----------------------------------|------|---|-----------------------------------|------|
| NaOH 1/50 N.....                      | 30.6  | 89                                |      |   |                                   |      | 54.4  | 92                                | 8.31 |
| 1/100 N.....                          | 30.6  | 89                                | 8.06 |   |                                   |      | 54.8  | 93                                | 8.16 |
| 1/200 N.....                          | 32.0  | 93                                | 8.02 |   |                                   |      | 56.3  | 96                                | 8.04 |
| Neut. Sal.....                        | 34.5  | 100                               | 7.96 | 48.4  | 100                               | 8.10 | 58.8  | 100                               | 8.02 |
| HCl 1/200 N.....                      | 34.0  | 99                                | 7.86 |   |                                   |      |   |                                   |      |
| 1/100 N.....                          | 34.1  | 99                                | 7.81 | 46.8  | 97                                | 7.89 |   |                                   |      |
| 1/50 N.....                           | 31.9  | 92                                | 7.68 | 45.7  | 95                                | 7.70 | 55.6  | 95                                | 7.65 |
| 1/33 N.....                           |   |                                   |      | 44.5  | 92                                | 7.55 |   |                                   |      |
| 1/25 N.....                           |   |                                   |      | 39.4  | 82                                | 7.34 |   |                                   |      |
| 1/20 N.....                           |   |                                   |      | 36.8  | 77                                | 7.06 | 48.5  | 83                                | 7.22 |

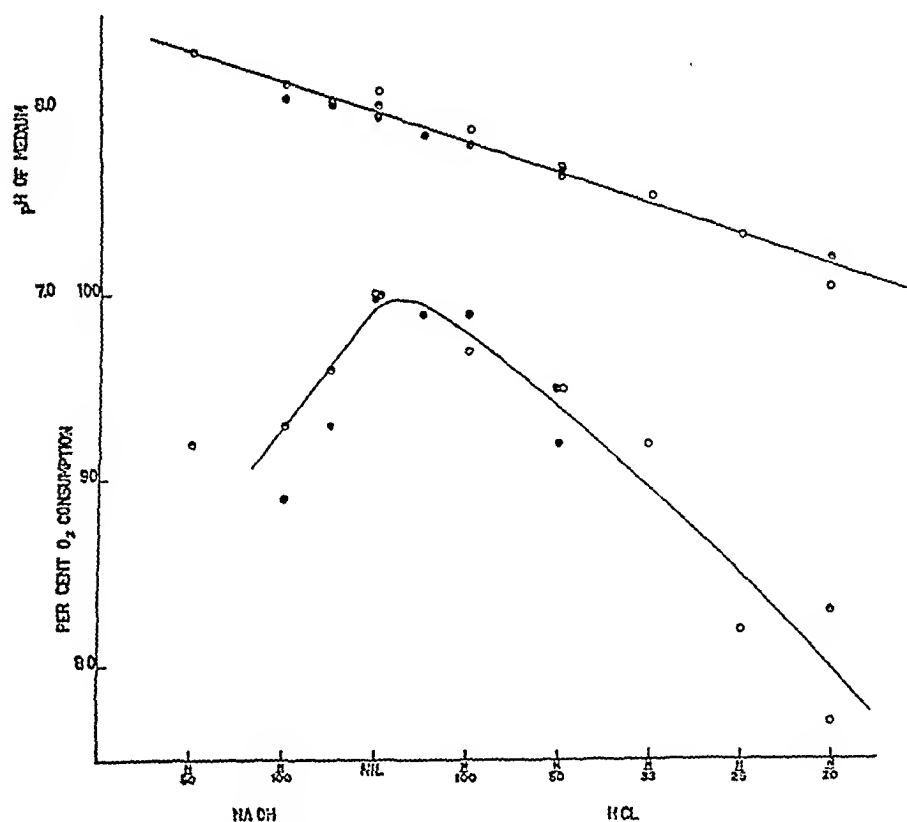


FIG. 2. Showing the influence of the addition of HCl and NaOH to blood upon its reaction and upon the respiration of the reticulocytes present in it.

The results of the experiments will be found in Table III and in Fig. 1.

It will be seen that variations in the reactions of the medium have a considerable influence upon the reaction of the cells. The maximum oxygen consumption occurred about pH 8.0, which would correspond to a reaction in the interior of the cells about pH 7.75. The metabolism at pH 7.4, or that normally to be found in the blood, would be about 85 per cent of this maximal value.

*The Influence of Variations in Tonicity upon the Respiration of Reticulocytes*

Determinations of the oxygen consumption of reticulocytes were made in solutions of sodium chloride of various concentrations ranging from 0.11 molar (0.6 per cent) to 0.28 molar (1.5 per cent). The solutions were added to the cells after they had been separated from their serum by centrifugation.

The results are given in the following table:

| Red cells in mills.<br>per c.mm. | NaCl: 0.6 per cent   | 0.9 per cent | 1.2 per cent | 1.5 per cent |
|----------------------------------|--|--------------|--------------|--------------|
|                                  | C.mm. O <sub>2</sub> consumed per billion reticulocytes per hour |              |              |              |
| 2.75 (14.2)*                     | 32.5   | 39.4         | 34.9         | 31.2         |
| 2.37 (23.4)                      | 38.5   | 44.2         | 39.6         | 37.8         |
| 1.96 (39.0)                      | 38.9   | 42.9         | 42.5         | 37.8         |

\* Reticulocyte percentages are given in brackets after the red cell counts.

It can be seen that in all three experiments the maximal respiration took place in a sodium chloride solution which was closely isotonic with normal blood. The figures, however, suggest that slight degrees of hypertonicity are less injurious to the cell respiration than the reverse condition of hypotonicity. The cells in 0.6 per cent saline have a metabolism 13 per cent below that in 0.9 per cent saline, while those in 1.2 per cent saline are only depressed to the extent of 7 per cent.

*The Influence of Glucose, Glycine, and Alanine on the Respiration of Reticulocytes*

In order to determine whether the presence of certain readily oxidizable substances in the surrounding medium resulted in any acceleration

tion of the rate of respiration of the reticulocytes, they were separated from their serum and added to saline solutions containing variable concentrations of these substances. Glucose was used in concentrations varying from zero to 400 mg. per hundred cc. Glycine and alanine were used in solutions containing from zero to 28 mg. of amino acid nitrogen per hundred cc. These amino acid solutions had no perceptible effect upon the rate of respiration, while the glucose solutions progressively depressed it, the respiration in saline containing 400 mg. per cent of glucose being on the average only 84 per cent of that in saline. Whether the glucose actually entered the reticulocytes is problematical. Kosawa (13), and Ege (14) found that the red cells of the rabbit are impermeable to glucose, but this may or may not be true of the reticulocytes.

Since reticulocytes have approximately the same rate of respiration in normal physiological saline solution as they have in their own serum, it is probable that the substances oxidized are present in the cells as a residue from an earlier stage of development, and are not gaining access to them continuously from the exterior. The disappearance of the reticulum during the period in which respiration takes place suggests that it is part if not the entire material consumed. The actual quantity of this substance oxidized may be approximately calculated. Since the reticulocyte stage of development is about 50 hours, and the average oxygen consumption about 60 cm. per billion reticulocytes per hour, the total quantity of oxygen used by every billion reticulocytes will be about 3 cc.

Unfortunately very little is known of the chemical nature of the reticular substance. Gawrilow (15), from a study of its reaction with vital stains, believes that it is partly protein, partly lipoid. Neither is there any consistent data upon the respiratory quotient of reticulocytes from which it might be possible to draw any conclusions as to its chemical constitution. Douglas (16) found an average respiratory quotient of 1.02 on three samples of citrated anemic rabbit's blood. This is much too high a value to be accounted for by the oxidation of either lipoid or protein, though there is no reason to believe that other substances may not be oxidized in addition to the vitally staining reticulum. Harrop and Barron (17) (1928) found values from which they suggested that the respiratory quotient was between 0.75 and

0.80, and such a value would of course be in closer agreement with Gawrilow's views.

Should the material consumed in the reticulocytes be a fatty acid, 3 cc. of oxygen would account for the oxidation of about 1.5 mg.; were it carbohydrate or protein, the weight would be greater. The weight of the dry material of one billion reticulocytes is about 40 mg., of which about 35 mg. is hemoglobin, so that the respiration of the reticulocyte must result in the destruction of a large proportion of its contents apart from the hemoglobin. This proportion is all the greater since more than 1 mg. of the residual 5 mg. is inorganic matter.

#### DISCUSSION

At the conclusion of this paper it is possible to compare the respiration of the mammalian reticulocytes and that of the normal nucleated and primitive red cells of the bird. Such a comparison is necessarily rough, but it may afford some indication of the solution of the problem which was raised at the commencement of the first paper of this series: the relative importance of the nucleus as the seat of cell oxidation. On the one hand, in the rabbit reticulocytes, we are concerned with a corpuscle entirely devoid of a nucleus; on the other, in the primitive avian erythroblast, a nucleated cell possessing an intensity of respiration not greatly inferior to that of a parenchymatous cell such as the liver cell.

Since all the determinations were made at the same temperature (37.5°C.) no allowance need be made for the temperature coefficients of the respiratory processes. There are, however, considerable differences in the sizes of the various cells whose respiration is to be compared. Consequently, in order to render the comparisons more satisfactory, the respirations should be considered not on the basis of cell number but rather upon one of cell volume. This is evident since the volume of the primitive red cell of the fowl is about twice as great as that of the rabbit's reticulocyte.

Hematocrit observations make it possible to determine the volume occupied by the various types of red cell. The volume of one billion red cells in the blood of anemic fowl is approximately 140 c.mm., in the blood of a normal fowl 100 c.mm., and of the reticulocytes of the rabbit 85 c.mm. In the blood of the anemic fowl about 50 per cent

of the cells were the normal variety of adult erythrocyte, so that the volume occupied by one billion of the more primitive cells would be about 180 c.mm. The following table shows the relationship between the respiration of these types of cells, together with their volumes, and allows a comparison to be made of their respiration on the basis of an arbitrary unit volume of 100 c.mm.

| Type of cell               | Volume occupied<br>by one billion<br>cells | Oxygen con-<br>sumption per<br>billion cells | Oxygen con-<br>sumption per<br>100 c.mm. cells |
|----------------------------|--|--|--|
|                            | <i>c.mm.</i>                               | <i>c.mm.</i>                                 | <i>c.mm.</i>                                   |
| Fowl: Prim. Red Cell. .... | 180  | 250  | 140  |
| Fowl: Norm. Red Cell. .... | 100  | 12   | 12   |
| Rabbit: Retics. ....       | 85   | 60   | 70   |

It is evident from this table that if the metabolism of the reticulocyte gives any indication of the intensity of oxidation taking place in the cytoplasm of a cell such as the primitive red cell of the fowl, that the oxidative processes of the cell are far from taking place exclusively in relation with the nucleus. The reticulum appears to be associated closely with the cell oxidation either as the material consumed or as the mechanism by means of which the process is effected. The possibility that the reticulum is in reality a nuclear remnant is now generally discredited. Both the primitive red cells of fowls and many of the normoblasts of mammals possess both a nucleus and a reticulum, and both are often present simultaneously in the cells as distinct structures. Further, their staining properties are dissimilar. It would seem probable, therefore, that in the primitive avian red cell oxidation processes are taking place both in the cytoplasm and in the nucleus and that the partition between the two may not be far from equal. Further, it is evident that though the definitive red corpuscles of the fowl are nucleated cells the respiration is small in comparison with the non-nucleated reticulocyte of the mammal with its residual cytoplasmic reticulum.

#### SUMMARY

1. The respiration of the reticulocytes of the rabbit has been measured during the period of an anemia produced by phenylhydrazine. Though the respiration increased greatly during the phase

of regeneration, the oxygen consumption per billion reticulocytes throughout the period remained approximately the same.

2. The respiration of the reticulocytes was affected by changes in the reaction of the medium in which they were suspended, and was at its maximum about a pH of 8, with a probable intracorporeal pH of about 7.75.

3. Variations in the tonicity of the suspending medium did not produce any great change in the respiration of the reticulocytes.

4. The presence of glycine, alanine, and glucose in the suspending medium resulted in no acceleration in the respiration of the cells. At higher concentrations glucose tended to depress the respiration. The material oxidized appears to be mainly or entirely contained in the corpuscles at the time they are liberated from the marrow.

5. A comparison is made of the respiration of the reticulated nucleated red cells present in the blood of anemic fowls and the non-nucleated reticulated red cells of rabbits. On the basis of equal volumes of cells, the respiration of the former is about twice that of the latter, while this in turn is about six times as great as the nucleated but non-reticulated normal red cells of the fowl.

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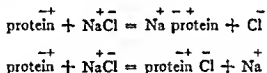
# THE EFFECT OF SALTS ON THE IONISATION OF GELATIN

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The internal salt, or "zwitter ion" theory of the structure of amino acids, has received increasing support of recent years. In the case of proteins, however, the data are less certain. According to the classical theory, gelatin is neutral at the isoelectric point by virtue of being either completely non-ionised or else containing a minimum number of dissociating groups, while the zwitter ion structure explains its behavior at this point on the basis of its possessing equal and maximal numbers of positive and negative charges. Such a molecule, with charges distributed over the surface, should be as capable of combining with a neutral salt as with an acid or base, and any such protein-salt compounds, since they involve at least one strong electrolyte, would be expected to be dissociated. Thus:



On the other hand, it is extremely difficult to postulate a mechanism whereby the neutral molecule of the classical theory could combine with neutral salt to produce a highly ionised compound, and even in the case of a molecule which bears a very small residual number of charges, any such combination could only be of a very slight order. The existence in considerable amounts of such compounds with neutral salts would therefore be indirect evidence for the zwitter ion structure of protein molecules.

The purpose of this paper is to draw attention to the fact that much of the data already published, particularly by Loeb and his co-workers, contain implicit evidence that the addition of neutral salts to



gelatin solutions increases the ionisation of the gelatin, *i.e.*, that the gelatin therefore combines with neutral salts to produce highly dissociated complexes.

Now if a gelatin solution be contained within a membrane permeable to electrolytes which is immersed in acid, it follows from the Donnan membrane relationship that the concentration of diffusible ions within the membrane is different from that outside it.

In the case of gelatin-HCl solution, on the acid side of pH 4.7, the H-ion concentration is less within the membrane than without, and the Cl-ion concentration is hence greater within the membrane, since

$$[H]_{in} [Cl]_{in} = [H]_{out} [Cl]_{out}$$

There is therefore an excess of the Cl-ions within the membrane, that is an excess of negative ions, and some of the gelatin must therefore be positively ionised to maintain electrostatic balance. If the H-ion activities are measured, the concentrations of all ions, including that of ionised gelatin, can be calculated. In the presence of known concentrations of salts the conditions are the same, and if the gelatin solution be at the same pH as when the salts are absent, then the comparison of the amounts of gelatin ionised in the two cases would give the effect of the addition of salt on the ionisation of gelatin.

Data on this point are readily available in the literature. The measurements at pH 3.8 are taken from Loeb (1) and Loeb and Kunitz (2). These determinations refer to the solutions at the beginning of the experiment, and in each case it has been necessary to assume that the pH of the outer solution has remained unchanged throughout. This is unavoidable since, not final pH measurements, but only membrane potentials, are given. Now the establishment of a membrane potential is merely the necessary consequence of a difference in concentration of H<sup>+</sup> or Cl<sup>-</sup> ions, from whatever cause, since:

$$E.M.F. = 59(pH_y - pH_x)$$

The agreement between the membrane potential observed and that calculated from the pH difference does not therefore possess any special significance, since it does not of itself confirm the Donnan relationship, but only checks the accuracy of the measurements. Even

if measurements of final pH within and without had been available, however, the correction to be made for the probable slight change in the pH of the outer solution would have been unimportant, since it is merely a difference of pH that is involved. Furthermore the volume of the outer solution was much greater than that of the inner and the pH change was therefore probably slight.

TABLE I

*Gelatin Chloride and Sodium Chloride. (1) and (2). 1 per cent Gelatin = 9 Milliequivalents Per Litre*

| Concentration of salt | In presence of sodium chloride; outer pH = 3.8 |            |                                  |  |                  |                     |                    | In absence of sodium chloride;<br>$\gamma_H = 0.99$ |                    |                  |                    |       |
|-----------------------|--|------------|----------------------------------|--|------------------|---------------------|--------------------|---|--------------------|------------------|--------------------|-------|
|                       | - log <sub>10</sub> molality                   | $\gamma_H$ | Membrane potential<br>millivolts | $C_{H^+}$ internal milli-<br>equivalents per litre | $CCl^-$ internal | $C_{Na^+}$ internal | $C_{Gelatin}$ ions | Membrane potential<br>millivolts                    | $C_{H^+}$ external | $CCl^-$ internal | $C_{Gelatin}$ ions | Ratio |
| M:8                   | 0.9  | 0.84       | 0                                | 0.189  | 125.0            | 125.0               | 0                  | 32.5  | 0.581              | 2.11             | 1.95               |       |
| M:16                  | 1.2  | 0.87       | 0.2                              | 0.181  | 63.20            | 62.00               | 1.02               | 32.5  | 0.577              | 2.10             | 1.94               | 0.52  |
| M:32                  | 1.5  | 0.90       | 1.0                              | 0.172  | 32.16            | 30.53               | 1.46               | 32.5  | 0.567              | 2.05             | 1.90               | 0.77  |
| M:50                  | 1.7  | 0.90       | 2.0                              | 0.164  | 21.62            | 18.66               | 2.80               | 33.0  | 0.542              | 1.97             | 1.82               | 1.54  |
| M:100                 | 2.0  | 0.92       | 3.5                              | 0.152  | 11.54            | 8.81                | 2.58               | 33.5  | 0.518              | 1.90             | 1.76               | 1.47  |
| M:200                 | 2.3  | 0.95       | 6.5                              | 0.131  | 6.57             | 3.93                | 2.51               | 33.5  | 0.462              | 1.70             | 1.57               | 1.60  |
| M:400                 | 2.6  | 0.97       | 11.0                             | 0.107  | 4.11             | 1.62                | 2.38               | 33.0  | 0.384              | 1.40             | 1.30               | 1.83  |
| M:500                 | 2.7  | 0.97       | 13.0                             | 0.099  | 3.61             | 1.20                | 2.31               | 33.0  | 0.350              | 1.26             | 1.16               | 1.99  |
| M:1000                | 3.0  | 0.98       | 18.0                             | 0.082  | 2.34             | 0.497               | 1.76               | 33.0  | 0.280              | 0.99             | 0.914              | 1.93  |
| M:2000                | 3.3  | 0.98       | 24.0                             | 0.063  | 1.67             | 0.197               | 1.41               | 33.0  | 0.231              | 0.846            | 0.783              | 1.80  |
| M:4000                | 3.6  | 0.99       | 29.0                             | 0.050  | 1.30             | 0.079               | 1.18               | 32.5  | 0.182              | 0.656            | 0.606              | 1.93  |
| M:8000                | 3.9  | 0.99       | 31.0                             | 0.048  | 0.955            | 0.037               | 0.870              | 32.5  | 0.172              | 0.621            | 0.573              | 1.52  |
| M:16000               | 4.2  | 1.0        | 33.0                             | 0.044  | 0.811            | 0.017               | 0.749              | 32.0  | 0.162              | 0.596            | 0.552              | 1.36  |

The first section of Table I gives the salt concentration and observed membrane potentials, the outer solution being at pH 3.8. From the membrane potentials were calculated the H-ion activities within the membrane, and from these the H-ion concentrations by use of Lewis and Randall's figures (3) for the activity coefficients of the H-ions in solutions of various strengths (Column 3), neglecting, of course, the unknown contribution of the gelatin to the ionic strength of the solution. The concentrations of the other ions, and therefore of the

gelatin which must be ionised to produce electrostatic equilibrium, follow. The second section gives the figures for a gelatin solution at the same pH as in the first section, but in equilibrium with HCl only. The last column shows the value of the ratio:

$$\frac{\text{Gelatin ionised in presence of NaCl.}}{\text{Gelatin ionised in absence of NaCl at same pH.}}$$

This ratio is plotted in the curve, the abscissae being the negative logarithms of the molality of the salt solution; this scale is simpler than plotting direct reciprocals of molality and corresponds closely to the pH scale.

The figures show clearly that apart from the depression of ionisation produced by very concentrated salt solutions in acid, the addition of salt to the gelatin-HCl or gelatin-NaOH systems increases the ionisation of the gelatin. The variation of the ionisation ratio in the pH range 3.8 to 4.3 reaches a maximum corresponding to about M/1000 NaCl solution. The curve is, however, the resultant of two effects, (a) of decreasing salt concentration from M/8 to M/16000 and (b) of increasing pH from 3.8 to 4.3, and at constant pH would be slightly flatter.

The effect of salts in depressing the osmotic pressure, viscosity and swelling of gelatin, as shown by Loeb, is to be considered quite apart from this increase in ionisation of the gelatin, which is most simply explained on the formation of complex salts.

The observations of Pauli (4) and (5) on electrolyte-free proteins, show from conductivity and ionic mobility measurements that proteins combine with zinc chloride to produce complex zinc-protein ions, positively charged, and also, under certain conditions, to give complex chlorine-protein ions. Northrop and Kunitz (6) also find that gelatin combines readily with calcium, copper and other salts to produce positively charged particles, and that the combination even close to the isoelectric point, becomes, in high salt concentrations, stoichiometrically equivalent to the combination with hydrogen.

In this connection it is possible to show that gelatin possesses an effective combining power toward metal ions of from 2.5 to 3.6, for the formation of complex ions under the conditions of the experiment. In Table II are given the concentrations, in millimols per 1000 gm.,

for Donnan systems of gelatin with calcium and copper chlorides. Column 1 gives the external concentration of the salt, as given by Northrop and Kunitz, and the second and third columns give the in-

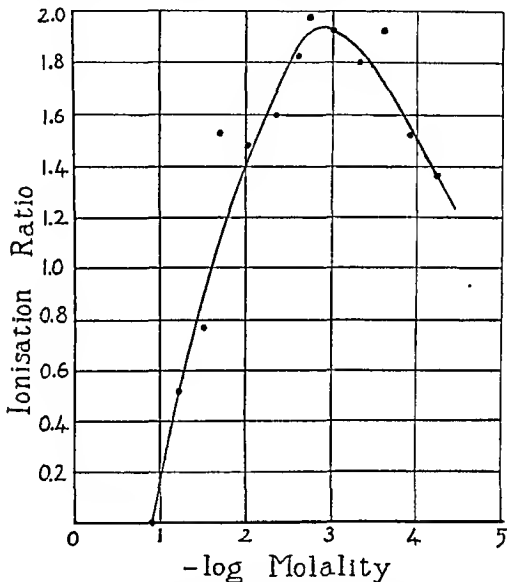


FIG. 1. Ionisation of gelatin produced by adding salt solution, from data of Loeb and Kunitz. The ratio of the amount of gelatin ionized in presence of NaCl to the amount ionized without NaCl but at the same pH is plotted against concentration of NaCl solution.

ternal concentrations of chloride and metal ions calculated from the membrane potential. Just as in Table I, there should be an excess of negative ions, and this is given by  $\alpha$  (Column 6). Now the amounts of copper found,  $[\text{Cu}]_2$ , are in excess of the theoretical by a consider-

able amount,  $[\text{Cu}]_3$ , which, since it is not subject to the Donnan relationship, cannot be present in the normal ionic form. It can only be present in association with the gelatin that has to be positively

TABLE II  
*Gelatin and Copper Chloride, pH 5.0\*\**

| $\text{C}_{\text{Cu}}$ external | $\text{C}_{\text{Cl}}$ internal | $\text{C}_{\text{Cu}}^{++}$ internal<br>calculated<br>$= [\text{Cu}]_1$ | $\text{C}_{\text{Cu}}^{++}$ internal<br>found $= [\text{Cu}]_2$ | Excess $\text{Cu}^{++}$<br>$= [\text{Cu}]_3$ | $\frac{\text{C}_{\text{Cl}}}{2} -$<br>$[\text{Cu}]_1 = a$ | Effective<br>combining<br>power $=$<br>$\frac{[\text{Cu}]_2}{a} \times 2$ |
|---------------------------------|---------------------------------|---|---|--|---|---|
| 8.8                             | 19.6                            | 7.1   | 10.7  | 3.6  | 2.7   | 2.67  |
| 8.05                            | 21.97                           | 4.32  | 13.15   | 8.83   | 6.66  | 2.65  |
| 187.9                           | 391.5                           | 173.1   | 204.6   | 31.5   | 22.7  | 2.77  |
| 178.0                           | 360.0                           | 174.0   | 184.5   | 10.5   | 6.0   | 3.50  |
| 192.5                           | 389.3                           | 188.4   | 199.5   | 11.1   | 6.3   | 3.52  |
| 485.0                           | 976.0                           | 479.0   | 495.5   | 16.5   | 9.0   | 3.66  |

*Copper Chloride + Deaminized Gelatin, pH 4.1†*

|       |        |       |       |      |       |      |
|-------|--------|-------|-------|------|-------|------|
| 191.8 | 387.1  | 188.4 | 194.1 | 5.7  | 4.7*  | 2.46 |
| 291.2 | 584.7  | 287.9 | 294.0 | 6.1  | 4.1*  | 2.97 |
| 383.9 | 771.4  | 380.4 | 386.5 | 6.1  | 4.9*  | 2.49 |
| 500.0 | 1014.0 | 487.0 | 515.0 | 28.0 | 19.6* | 2.86 |

*Gelatin + Calcium Chloride, pH 5.0‡*

| $\text{C}_{\text{Ca}}$ external | $\text{C}_{\text{Cl}}$ internal | $\text{C}_{\text{Ca}}^{++}$ internal<br>calculated<br>$= [\text{Ca}]_1$ | $\text{C}_{\text{Ca}}^{++}$ internal<br>found $= [\text{Ca}]_2$ | Excess $\text{Ca}^{++}$<br>$= [\text{Ca}]_3$ | $\frac{\text{C}_{\text{Cl}}}{2} -$<br>$[\text{Ca}]_1 = a$ | Effective<br>combining<br>power<br>$=$<br>$\frac{[\text{Ca}]_2}{a} \times 2$ |
|---------------------------------|---------------------------------|---|---|--|---|--|
| 295.0                           | 594.0                           | 291.0   | 299.0   | 8.0  | 6.0   | 2.67   |
| 494.0                           | 992.0                           | 490.0   | 501.0   | 11.0   | 6.0   | 3.67   |
| 744.5                           | 1495.0                          | 739.0   | 751.0   | 12.0   | 8.5   | 2.82   |
| 984.0                           | 1976.0                          | 976.6   | 994.5   | 17.9   | 11.4  | 3.16   |

\* Values corrected for concentration of  $\text{H}^+$  ion.

\*\* See Table I, Northrop and Kunitz (6).

† See Table II (6).

‡ See Table VII (6).

ionised to neutralise the excess of negative ions. In the first case, the excess of divalent negative ions is 2.7 millimols, which must therefore be neutralised by 2.7 millimols divalent positive ions. The excess of

copper over the theoretical is 3.6 millimols, and these must therefore be absorbed into the formation of 2.7 millimols divalent positive Cu-gelatin ions, so that the gelatin must be acting as though  $\frac{3.6}{2.7} \times 2$ -valent. This effective valency or combining power of the gelatin for complex ion formation is given in the last column, and is seen to increase slowly with increasing concentration of salt, from a value of 2.67 upwards. Calcium chloride yields similar results; deaminised gelatin shows a lower combining power. In this last case correction has to be made for the H-ion concentration; at pH 5.0 this is negligible, at pH 4.1 it reduces the value of  $a$  by 0.4.

As in the previous tables, the figures represent small differences between large values, and the agreement is therefore very satisfactory. The formation of ionisable compounds by the addition of salts goes far to explain the solubility of the globulins in neutral salt solutions, since the idea, so often put forward, that the ions are soluble and the non-ionised protein is not, now receives a definite mechanism for the production of ions by salt. As Vickery and Osborne (7) point out, this fact has been somewhat of a mystery until now.

Furthermore, the formation of complex ions, while difficult to explain on the classical theories of protein structure, falls readily into line with the zwitter ion conception and is in fact an expected corollary therefrom.

#### SUMMARY

The effect of the addition of sodium chloride to gelatin solutions is shown from the Donnan relationship to increase the ionisation of the gelatin, the increase produced in acid solutions reaching a maximum at about 1/1000 molar salt concentration. This effect is attributed to the formation of complex ions.

From the similar action of calcium and copper chlorides the effective combining power of gelatin for complex positive ion formation is deduced. The bearing of complex ion formation on the zwitter-ionic structure and solubility phenomena of proteins is pointed out.

The author's thanks are due to Dr. M. Kunitz for directing his attention to some of the published measurements, to H. M. Winegar-

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# THE KINETICS OF THE BACTERIUM-BACTERIOPHAGE REACTION

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The clearing of broth cultures of bacteria by an agent which produces more of itself during the period of contact with susceptible organisms constitutes a striking phenomenon, a process obviously of great interest to bacteriologists and one which accordingly has been the subject of extensive study. However, attempts to analyze the mechanism of phage action upon bacteria have not resulted in general concordant interpretation of the basic experimental facts, and indeed many of the major facts themselves are wanting or are in dispute. This is due in part to the inaccuracies involved in determining phage quantitatively and to the difficulty of estimating bacteria in the presence of phage. One of us has described experimental procedures for determining phage (1) and for estimating bacteria in phage-containing suspensions (2), both being sufficiently convenient and accurate for use in a quantitative analysis of the phage-bacterium system. The present paper deals with such an analysis in the particular case of an antistaphylococcus bacteriophage acting upon a single strain of *S. aureus*.

## *General Description of the Reaction between Antistaphylococcus Bacteriophage and S. aureus*

Before going on to the reaction itself the terms and abbreviations used throughout should be defined.

*B*: Bacterium, a 16 hour agar culture of a single phage-susceptible strain of *S. aureus* suspended in saline.

[*B*]: Total number of bacteria/ml.

*Bo*: Initial value of [*B*].

*Be*: [*B*] at maximal stationary phase of *B* growth.



*P*: A polyvirulent highly active antistaphylococcus phage.

P.U.: Phage unit.

[*P*] or [P.U.]: Phage units/ml.

*P*<sub>0</sub>: Initial value of [*P*].

Broth: Veal infusion, 1 per cent peptone, 0.5 per cent NaCl, pH 7.6.

Standard phage: Antistaphylococcus bacteriophage prepared by suspending  $2 \times 10^7$  staphylococci per ml. in 1 per cent phage.

The mixture is rocked at 36°C. until lysis is complete. The lysate is stored at 5°C. without filtering.

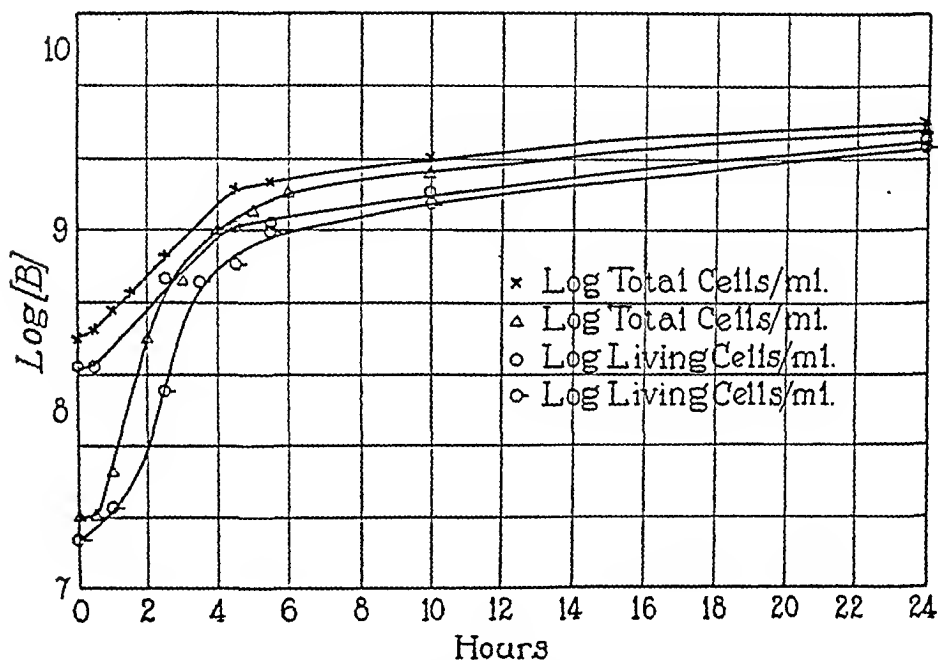


FIG. 1. Normal growth curves of *S. aureus* in broth at 36°C. Plots of log [*B*] against *t* for two different values of *B*<sub>0</sub>. Total cells estimated by turbidity method, living cells by plate count.

For each experiment the following data were obtained at various time intervals: Total *P*/ml.; extracellular *P*/ml.; intracellular *P*/ml. (by difference); *B*/ml., in the *P*-containing suspension and also in the broth alone without *P*. Essential conditions prevailing unless otherwise indicated were: Temperature 36°C. and constant agitation obtained by placing the test series in a mechanical shaker mounted in a water bath. The methods employed in making the various deter-

minations are described at the end of the paper. The complete data furnish the basis upon which the following description of the *B-P* reaction rests.

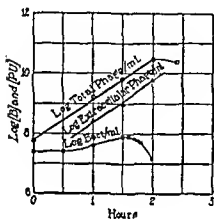


FIG. 2

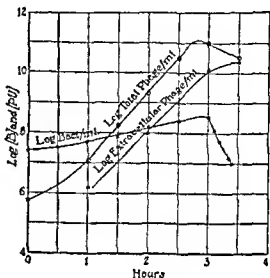


FIG. 3

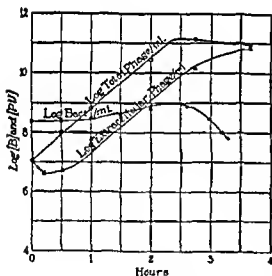


FIG. 4

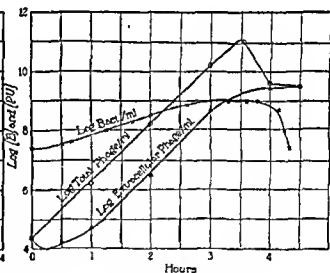


FIG. 5

FIGS. 2, 3, 4 and 5. Plots of typical kinetic experiments. The curves of log total  $[P]$ , log extracellular  $[P]$  and log  $[B]$  against  $t$  for different values of  $P_0$  and  $B_0$  at  $36^\circ\text{C}$ . are shown.

*A. Normal Growth.*—Normal growth of the test organism in broth at  $36^\circ\text{C}$ . is shown in the plot of log  $[B]$  against time (Figs. 1 and 11). The usual features of a growth curve are observed here, namely, a period of lag (average about 0.5 hour), a brief phase of positive

acceleration, a relatively long period of logarithmic growth, a phase of negative growth acceleration and a prolonged maximum stationary phase.

*B. Growth in the Presence of Phage.*—Bacteria grown in the presence of phage have failed to show any differences in their growth curves from those of broth cultures maintained under like conditions of temperature, pH, agitation, etc., except when the initial  $P/B$  ratio has been very high. That is, a normal growth curve is followed up to a certain point at which lysis begins explosively. There is no evidence that lysis is a continuous process from the time  $P$  and  $B$  are mixed or that  $B$  growth is stimulated by  $P$ . The special case of growth deviation seen in cultures containing very high  $[P]$ 's relative to  $[B]$  at the start of growth is considered below.

*C. Production of Phage Is Logarithmic.*—In Figs. 2, 3, 4 and 5 the plot of  $\log$  total  $[P]$  against  $t$  is a straight line rising to a maximum and dropping off thereafter. Since the growth of bacteria is also logarithmic at first, it follows that the ratio  $P/B$  increases logarithmically with time. Fig. 6, containing the plots of four experiments, indicates that this is the case. One cannot say then that every time a  $B$  divides a certain amount of  $P$  is formed. If this were so the  $\log$  plots of  $B$  and  $P$  against  $t$  would be parallel lines. Physically, the large value of  $d \log P/dt$  as compared to  $d \log B/dt$  signifies that the rate of  $P$  production is proportional to a power of the rate of  $B$  reproduction.

The curves of Figs. 2, 3, 4 and 5 show no conclusive evidence of a lag in  $P$  formation corresponding to the lag in  $B$  formation. Fig. 7 is a plot of two experiments performed particularly to detect a lag and both show very definitely a slight lag lasting between 0.4 and 0.5 hour. This would be overlooked in the ordinary run of experiments in which samples are taken at hourly intervals.

The view that  $P$  production is a process independent of bacterial lysis has been put forward by several investigators (3)–(13). Others, chief of whom is d'Herelle (19), feel that lysis is the essential part of bacteriophagy and that the accumulation of phage is dependent upon its liberation from lysing bacteria.

*D. Relation of Extracellular  $P$  to Total  $P$ .*—Figs. 2, 3, 4 and 5 contain plots of  $\log$  extracellular  $[P]$  against time. This represents the  $P$  content of the clear fluid left after centrifuging off  $B$ . Here again the

development of  $P$  is seen to be logarithmic, maintaining a constant ratio to the intracellular  $P$  as shown by the fact that the log plots of extracellular  $[P]$  and total  $[P]$  against  $t$  are parallel. Fig. 7 indicates that at 0.4 to 0.5 hour from the time of mixing  $P$  and  $B$  75 per cent of the  $P$  present in the mixture is within or adsorbed to the  $B$  while but 25 per cent is free in the supernatant fluid. Other experiments of this sort give values of 70 to 90 per cent for the first and 10 to 30 per cent for the second of these fractions. After 0.5 hour

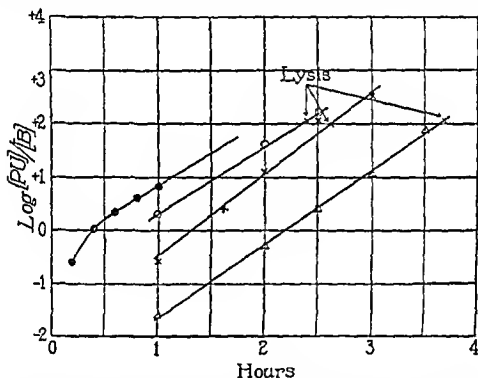


FIG. 6. The development of bacteriophage per organism with time in four separate experiments;  $B_0$  constant,  $P_0$  varying. The plots show that the formation of  $P$  per  $B$  is a logarithmic process; also that lysis results when  $\log P/B = 2.1$ .

and until the maximal  $[P]$  is approached there appears to be an equilibrium between the  $P$  associated with the  $B$  and that free in the suspension, *i.e.*, the free  $[P]$  is a constant low percentage of the total  $[P]$ . In this respect the  $P$  distribution is quite what one would expect in the case of a relatively simple chemical compound and not what would be anticipated if  $P$  is a comparatively complex organized body. As the total  $[P]$  approaches a maximum (Figs. 2-5) the curve of extracellular  $[P]$  flattens and the total  $[P]$  curve descends from a maximum to meet it, indicating some destruction of intracellular  $P$  during lysis of  $B$ .

*E. Growth of B Essential for Production of P.*—The logarithmic production of *P* during the log phase of *B* growth suggested that *P* formation might be intimately associated with the growth of *B*. Several experiments have been undertaken to investigate this point.

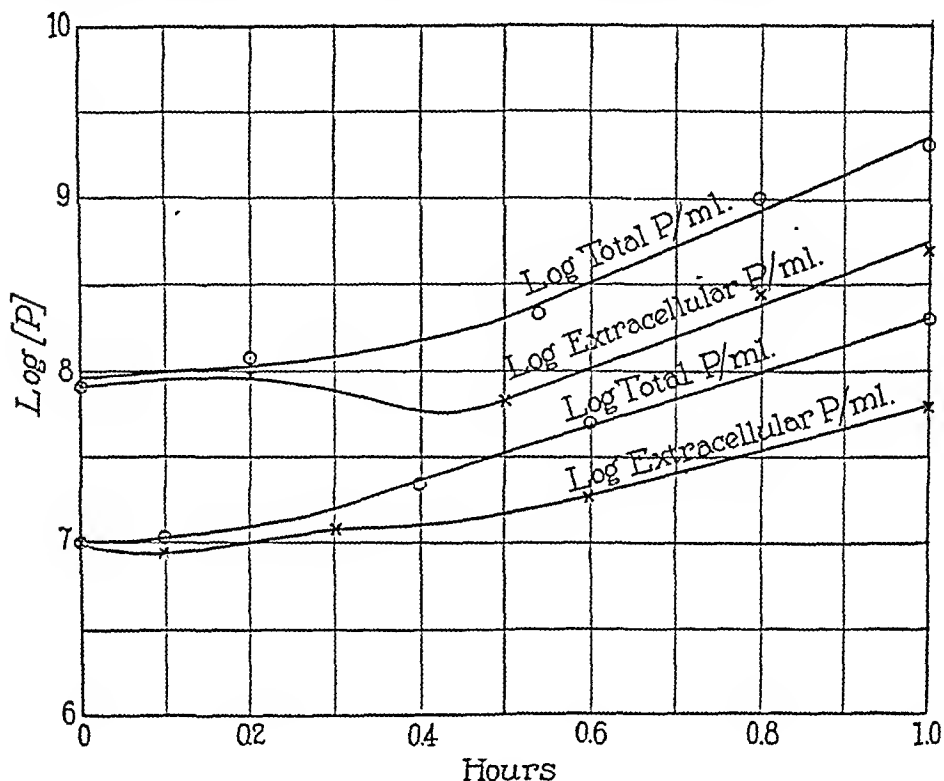


FIG. 7. Two experiments performed to detect lag in *P* formation and to establish the relationship between intracellular *P* and extracellular *P* early in the process of *P* production. A definite lag is evident, *P* production becoming logarithmic with *t* at 0.4 to 0.5 hour. By 0.5 to 0.6 hour the major percentage of total *P* has become intracellular *P*. The equilibrium between intracellular *P* and extracellular *P* established at this time is maintained up to  $t_{(lysis)}$ .

(a) *P* Formation in *P*-*B* Mixtures with Initial [*B*] Approaching  $B_c$  and with Initial [*B*] >  $B_c$ . *B* and *P* were mixed in broth in such amounts that the final respective concentrations were  $1 \times 10^{9.05}$  *B*/ml. and  $1 \times 10^{7.9}$  P.U./ml. The tubes were placed in the shaker at 36°C. and the *P* and *B* curves followed. The [*B*] was already near the limiting value of the maximum growth stationary phase (9.4) and the

growth curve (Fig. 8) showed merely a short phase of negative growth acceleration before reaching this  $[B]$ , after which no growth was detectable. The  $[P]$  meanwhile had increased one hundred times but as growth ceased the curve of  $P$  production flattened also. No demonstrable lysis occurred in this suspension and no further increase in  $[P]$  was noted. Fig. 8 represents another experiment of the same sort. Here the initial  $\log [B]$  was 9.7 (considerably  $> B_e$ ) and the corresponding  $\log [P]$  9.5. The mixture was kept at  $36^\circ\text{C}$ . in the shaker.

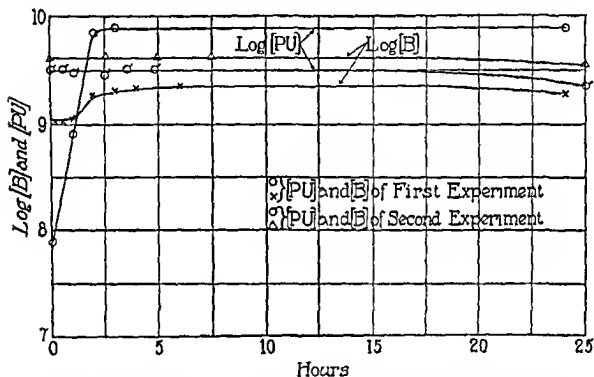


FIG. 8. Relationship between  $P$  formation and  $B$  growth. In Experiment 1  $B_0$  is not far from  $B_e$ , growth of  $B$  is limited and  $P$  production occurs only during the short period required for  $B_0$  to rise to  $B_e$ . In Experiment 2  $B_0 > B_e$ , there is no  $B$  growth and no  $P$  formation.

No growth of  $B$  took place and there was no production of  $P$  during 24 hours. That the cells were living was demonstrated by plate counts on controls at the end of the experiment.

(b) *P Formation in P-B Mixtures Made with Limited Supply of Nutrient Material and Kept at Moderately Low Temperature to Retard B Growth. Effect of Increasing Nutrients and Raising Temperature.*—Standard  $P$  was diluted 1–1,000 with saline. To 4 ml. of this dilution,  $B$  suspended in saline were added. The final concentrations were

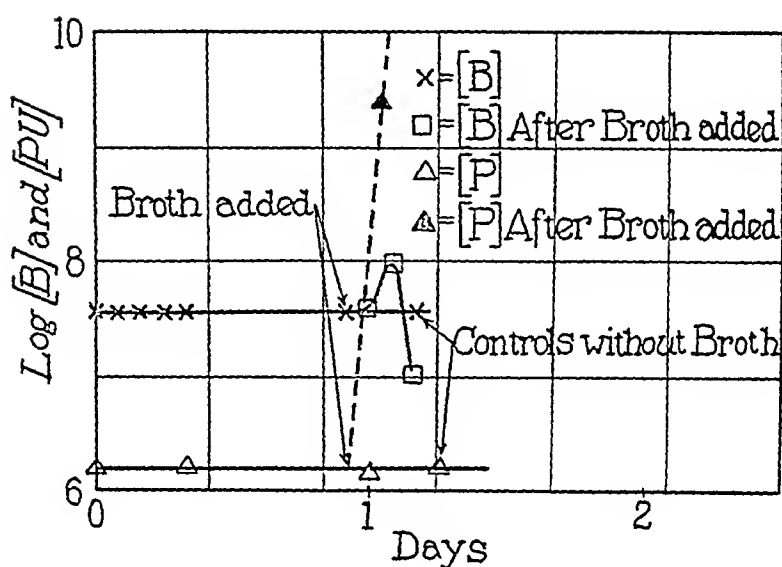


FIG. 9. Relationship between  $P$  formation and  $B$  growth.  $B$  growth inhibited by low concentration of nutrients and moderately low temperature. No  $P$  formation occurs until broth added and temperature raised permitting  $B$  growth.

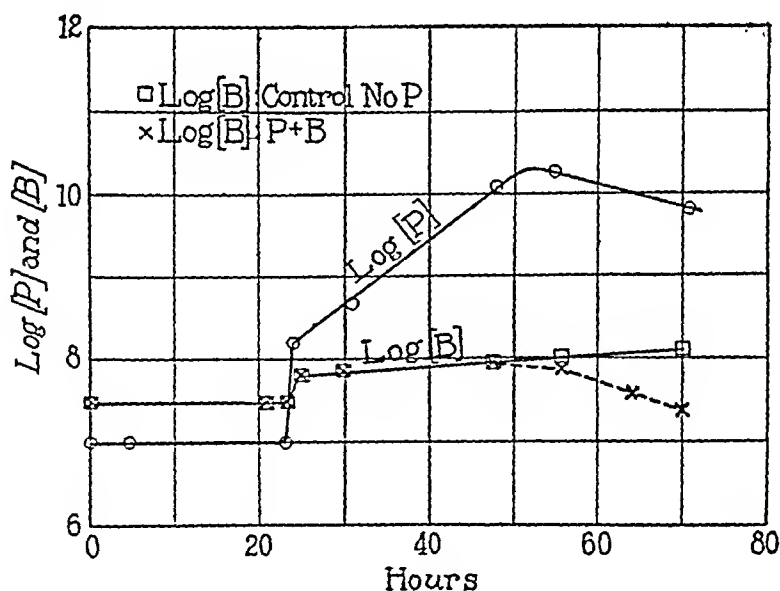


FIG. 10. Relationship between  $P$  formation and  $B$  growth.  $B$  growth retarded by low temperature ( $10^{\circ}\text{C}$ ).  $P$  production proceeds only when  $[B]$  is increasing. Lysis occurs when  $\log P/B = 2.1$ .

respectively  $1 \times 10^{6.2}$  P.U./ml. and  $1 \times 10^{7.6}$  B/ml. The amount of nutrient material was greatly reduced to inhibit growth of *B*; to further retard growth the tubes were kept at 21°C. Determinations of *P* and *B* at intervals indicated no rise in [*B*] or [*P*] up to 23 hours [Fig. 9). At this time 1 ml. of broth was added to each of several tubes and they were placed in the shaker at 36°C. As controls some of the original tubes without the addition of broth were shaken at 36°C, and others were kept at 21°C. The tubes receiving broth at once showed growth of *B* and the concomitant production of *P*. Lysis occurred as usual. In the control tubes no growth and no production of *P* were observed.

(c) *P* Formation during Retarded *B* Growth at Very Low Temperature.—Into a series of tubes *P* and *B* were pipetted in such amounts that final concentrations of  $1 \times 10^7$  P.U./ml. and  $1 \times 10^{7.4}$  B/ml. were attained. The tubes were maintained at 10°C. in a shaker. Fig. 10 indicates no bacterial growth for 22 hours. Likewise no *P* was formed. However, when *B* growth once began *P* production was also noted. Lysis eventually took place.

(d) *P* Formation When *B* Is Artificially Raised.—If the assumption that *P* is produced only during *B* growth is correct then any means of inducing the *B* of a *P*-*B* mixture to grow beyond the level of the usual maximum stationary phase should result in more than the ordinary amounts of *P* being formed.

5 ml. of broth containing  $1 \times 10^{9.4}$  B/ml. were added to 5 ml. of *P* containing  $1 \times 10^{10}$  P.U./ml. The mixture was dialyzed against broth through an acetic collodion membrane of such porosity that *B* and *P* were retained while metabolites and broth might be interchanged. A control of *B* in broth reached a value of  $1 \times 10^{9.3}$  B/ml. before growth ceased. In the *P*-*B* mixture the *B* rose to  $1 \times 10^{9.7}$ /ml. and the *P* to  $1 \times 10^{11.9}$  P.U./ml., a [*P*] some one hundred times that obtained in the usual lysate. Lysis occurred at this point.

In all these experiments then it was not possible to produce *P* without growth of *B*, indicating that *P* production is somehow conditioned by *B* growth. This intimate relationship between *B* growth and *P* production has been stressed in earlier work (4, 11, 14-18).

*F. Lysis Occurs When  $\log P/B = 2.1$  (Ratio P.U./B = 125).*—So far in this discussion there has been no reference to the mechanism



initiating lysis of bacteria. Turning again to Figs. 2, 3, 4 and 5, it is evident that the decrease in  $[B]$  begins when the total  $[P]$  reaches a point 2.1 on the log scale above the maximal  $[B]$ . Assuming for the moment that this relationship is significant and that lysis will begin in any culture when the ratio of  $P/B = 125$  or  $\log P/B = 2.1$ , it is obvious that those  $P$ - $B$  mixtures in which the original  $[P]$  is low com-

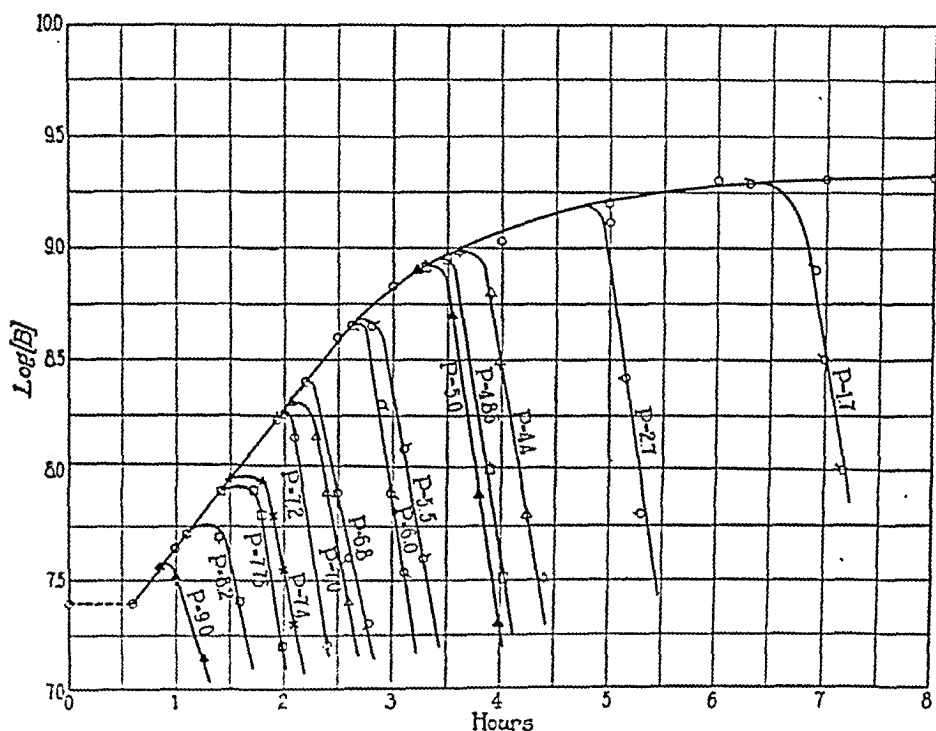


FIG. 11.  $B$  growth in broth and in  $P$ - $B$  mixtures made with  $\log B_0 = 7.4$  and various  $P_0$ 's (temperature  $36^\circ\text{C}$ .). The growth curves are identical until lysis begins.  $t_{(17.5)}$  obviously depends upon  $P_0$ . The process of lytic destruction of  $B$  is seen to be logarithmic with  $t$  and the values  $-dB/dt$  are constant for widely varying  $P_0$ 's.

pared to  $[B]$  will require a longer time to lyse than those having an original  $P/B$  ratio closer to the limiting value. This is experimentally true. The assumption will also explain the lack of growth or at most very slight growth seen in cultures to which have been added very high  $[P]$ 's as in the first curves of Fig. 11. Here the initial values of  $\log P - \log B$  are  $9 - 7.4$ ,  $8.2 - 7.4$  and  $7.75 - 7.4$  at the start of the

reaction. In the first instance lysis occurs very quickly for the ratio  $P_0/B_0$  is near the critical value, in the second case there is slight growth until enough  $P$  has been produced to reach the limiting ratio and in the third instance even more bacterial growth is required.

So far then the assumption is qualitatively in accord with experimental facts. A further prediction is that any culture of bacteria will lyse without further growth of  $B$  if we add to it 125 p.u. for each  $B$ . The following experiments serve to substantiate the hypothesis and to

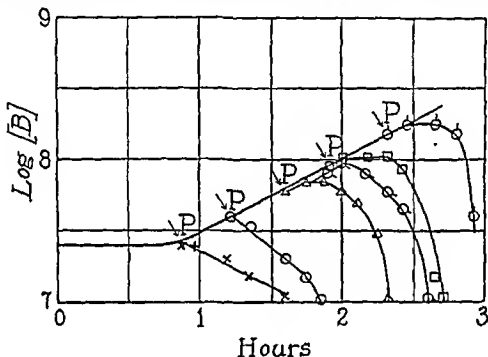


FIG. 12. Lysis is conditioned upon the value  $\log P/B = 2.1$ .  $4 \times 10^{10}$  p.u. (vol. 4 ml.) were added to tubes of growing  $B$  (vol. 4 ml.) at double the  $[B]$ 's indicated (volume doubled by  $P$  addition and  $[B]$  halved). Lysis without growth occurred when  $\log P/B = 2.1$  or more. Below this value  $B$  growth proceeded until enough  $P$  was produced to reach the critical value.

stress the significance of the  $P/B$  ratio as an essential condition for the initiation of lysis.

(a) *Lysis without Further  $B$  Growth Follows the Addition of  $P$  to  $B$  in Amounts Effecting a  $P/B$  Ratio of 125 or More.*—4 ml. aliquots of  $B$  in broth ( $2.5 \times 10^7$  B/ml.) were grown at  $36^\circ\text{C}$ . in the shaker. At various points on the growth curve 4 ml. of  $P$  (titre  $1 \times 10^{10}$  p.u./ml.) were added to the  $B$ . The course of events after the addition of  $P$  was followed and the results plotted (Fig. 12). Lysis began imme-

diately in two instances, namely, when the  $P/B$  ratios at the time of  $P$  addition were 200 and 125. The addition of  $P$  at points higher than this on the  $[B]$  curve did not result in immediate lysis; rather growth

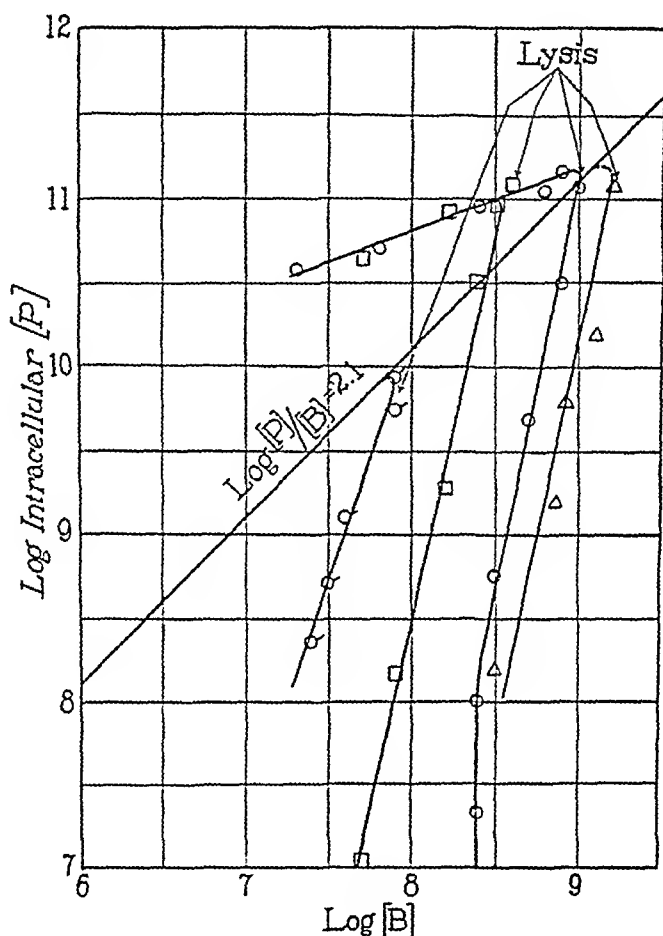


FIG. 13. Plot of log intracellular  $[P]$  against log  $[B]$ . Lysis occurs when intracellular  $[P]/[B] = 2.1$ . This value holds for the intracellular  $P/B$  ratio as well as for total  $P/B$  since intracellular  $P$  constitutes the major percentage of total  $P$ .

continued with the concomitant production of  $P$  until enough was formed to attain the critical  $P/B$  ratio.

(b) *Lysis at Low Temperature Occurs When  $\text{Log } P = \text{Log } B + 2.1$ .*—The experiments done at  $10^\circ\text{C}$ . show the same general trend, i.e., a  $P/B$  ratio of approximately 125 must be reached before lysis begins (Fig. 10).

The experimental data point definitely then to the attainment of a particular  $[P]$  per  $B$ , and not to a critical  $[P]$  per ml., as the condition responsible for the lytic process. It cannot be said at this time, however, whether the ratio intracellular  $P/B$ , extracellular  $P/B$  or total  $P/B$  is the one involved. If a series of  $P$ - $B$  mixtures are centrifuged at intervals shortly after the addition of  $P$  to  $B$  it is possible to separate the cells from  $P$  at various stages of the process of  $P$  diffusion into (or adsorption to)  $B$ . By observing the  $t_{(lysis)}$  of such bacteria resuspended in broth and comparing with controls resuspended in the supernatant  $P$  it might be expected that some information as to the relative rôles of the intracellular and extracellular  $P$  fractions in the instigation of lysis could be elicited. Experimentally, the cells separated shortly after mixing  $P$  and  $B$  require considerably longer for the onset of lysis than those centrifuged from  $P$  when equilibrium between intracellular and extracellular  $P$  has become established (0.5 hour). The removal of the extracellular  $P$  after this time causes no change in  $t_{(lysis)}$ . Further, if  $P$ - $B$  mixtures are centrifuged shortly before lysis is expected to occur and the  $B$  are resuspended in broth, lysis commences at the same time in these tubes and in control suspensions centrifuged and resuspended in the supernatant  $P$ .

This all might be interpreted as stressing the importance of intracellular  $P$  in bringing about  $B$  lysis were it not for the following facts: When equilibrium between intracellular and extracellular  $P$  once has been attained the value of extracellular  $[P]$  is very small compared to intracellular  $[P]$ . Therefore any difference in  $t_{(lysis)}$  occasioned by its removal is quite small and probably is within the limits of experimental error. Also, shortly after resuspending the centrifuged cells in broth at any time after intracellular-extracellular  $P$  equilibrium has become established the extracellular fraction quickly appears again and equilibrium is maintained up to  $t_{(lysis)}$ . The significance of the longer  $t_{(lysis)}$  noted in the case of cells centrifuged and resuspended in broth soon after the addition of  $P$  to  $B$  is likewise open to question. It is known that but a small percentage of total  $[P]$  comes down with the  $B$  at this time; again, extracellular  $P$  is present immediately after the  $B$  are resuspended in broth. Consequently the difference in  $t_{(lysis)}$  may well be due to the lower value of total  $[P]$  or extracellular  $[P]$  and not to the smaller intracellular  $[P]$ .

Obviously this type of experiment leads to no definite conclusions beyond the one established point that lysis follows the attainment of a particular  $P/B$  ratio. Empirically and rationally any one of the ratios intracellular  $[P]/[B]$ , extracellular  $[P]/[B]$ , or total  $[P]/[B]$  might represent the prime conditioning agent for lysis.

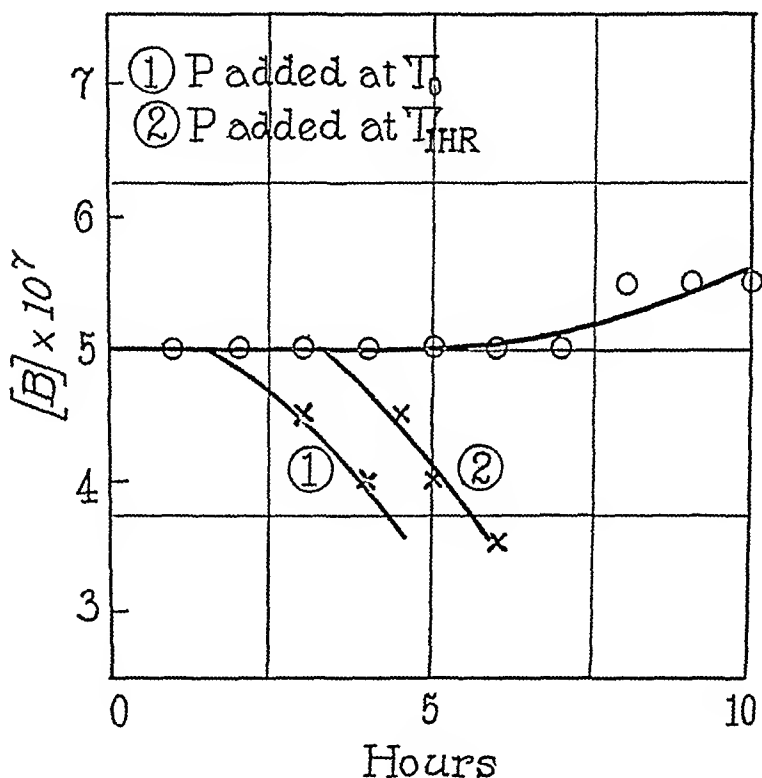


FIG. 14. *B* growth is not essential for lysis. *P* added to *B* during prolonged lag phase of *B* growth induced by low temperature ( $12^\circ\text{C}$ ).  $\log P/B = 2.2$  in both instances. Lysis occurred before *B* growth became evident in controls of *P-B* mixtures.

*G. Growth of B Is Not Essential for Lysis.*—The question of whether or not *B* must be in the growth phase for lysis to occur is a difficult point to investigate experimentally. *B* which have reached the maximum stationary phase cannot be used because the addition of *P* dilutes the suspension below the maximum  $[B]$  and growth begins again. If the *B* are centrifuged from a fully grown culture and are

made up to the maximal stationary  $[B]$  in  $P$ , the  $[P]$  attainable will still be below the critical  $P/B$  ratio because  $\log [P]$  of standard undiluted  $P$  is only 10.0 and  $Be = 9.4$ . Consequently, one is forced to inhibit growth by some artificial means, chemical or otherwise. It seemed best in the present case to avoid complications inherent in the addition of chemicals to the  $P$ - $B$  system and rather to make use of the fact that the lag phase of  $B$  growth at low temperatures is very markedly prolonged.

(a) *B Can Undergo Lysis during the Lag Phase Before Growth Begins.*—Tubes containing a total of  $25 \times 10^7 B$  in 1 ml. of broth were placed in the water bath shaker at  $12^\circ\text{C}$ . At various times 4 ml. portions of  $P$  (titre  $1 \times 10^{10}$  p.u./ml.) were added to the  $B$  suspensions, giving a  $P/B$  ratio of 160. Controls of  $B$  alone without  $P$ , both concentrated and dilute, were run at the same time. The  $B$  curves are given in Fig. 14 and indicate quite clearly that lysis had started before growth began.

From this experiment and the preceding ones it seems justifiable to conclude that the essential condition for initiation of lysis is a minimum  $P/B$  ratio of 125 and that  $B$  need not be growing for lysis to occur. This conclusion is made more tenable by the fact that lysis of cultures seeded with very small  $Po$ 's occurs when the growth curve is definitely well within the maximal stationary phase (Fig. 11) (a common observation).

*H. Lytic Destruction of B Is a Logarithmic Process.*—From the graphic representation of  $[B]$  during lysis of a  $B$  suspension (Fig. 11) by various initial  $[P]$ 's it is apparent that  $B$  destruction is logarithmic with time, in this respect being analogous to most death rate processes. Further, the rate at which lysis proceeds with a given initial  $[B]$  is constant for widely varied  $Po$ 's. The temperature coefficient of the rate of lysis is remarkably high (compare Figs. 10 and 11).

*I. The Major Portion of P Present at End of Bacteriophagy Is Set Free from Lysing Bacteria.*—The plots of Figs. 2, 3, 4 and 5 are logarithmic and give a false impression of the conditions actually obtaining during lysis. Fig. 15 represents two sets of the same data plotted on an arithmetic scale. The lower points are of course meaningless because of the large scale used, but the curves during the time of lysis show clearly that 30 to 50 per cent of the maximal total  $[P]$  or 40 to

60 per cent of the maximal intracellular  $[P]$  formed in the course of the reaction are lost as the  $B$  are destroyed. Since at the maximum  $[P]$

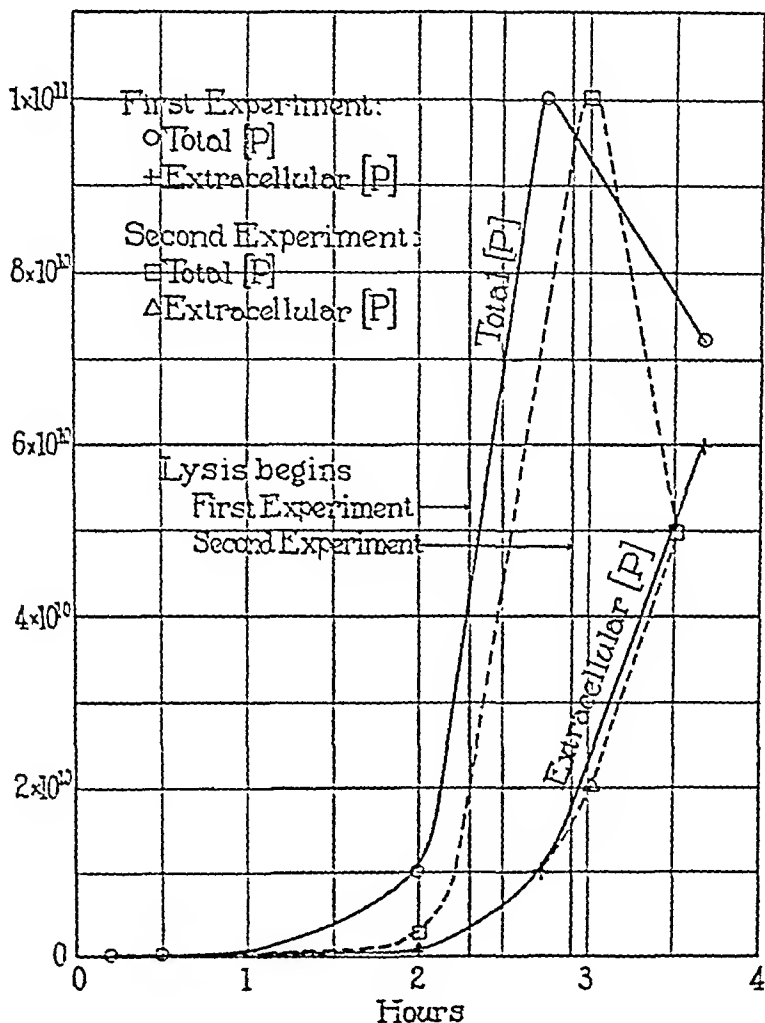


FIG. 15. An arithmetic plot of total  $P$  and extracellular  $P$  against  $t$  in two different  $P$ - $B$  mixtures to show relationship existing at the development of maximal  $[P]$  and  $t_{(1/2)}$ .

attained, intracellular  $P$  constitutes the major percentage of total  $[P]$ , it is probable that the chief loss in  $P$  during lysis takes place in the intracellular fraction.

*J. End Titre Values after Bacteriophagy.*—When the entire process of bacteriophagy is completed the lysate is left with a certain  $[P]$  determined by the now completed  $P$ - $B$  reaction and maintained at a constant level for long periods of time if the phage is stored at low temperatures. These final titre values for various initial values of  $[B]$  and  $[P]$  fall within a relatively narrow range. The explanation of this lies in the large value of  $dP/dt$  as compared to that of  $dB/dt$ . Fig. 16 is a plot of  $B$  growth against time and with it are curves of  $P$

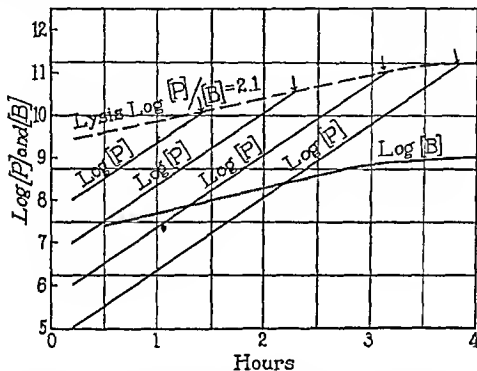


FIG. 16. A plot of  $\log [B]$  against  $t$ , with plots of  $\log [P]$  against  $t$ . The latter curves originate in various  $Po$ 's and are assigned an average slope. The breaks at the origin allow for average lags in  $P$  and  $B$  development. Lysis is seen to occur when  $\log [P]/[B] = 2.1$ .  $t_{(17.12)}$  is predicted fairly accurately (cf. Fig. 11).

formation to which have been assigned the average slope of numerous experimental curves. Clearly, on all the  $\log [P]$  curves originating in widely varying  $Po$ 's the points at which  $\log [P] = 2.1 + \log B$  (lysis begins) lie within a narrow zone of  $\log [P]$  values (10–11). That is, the maximal  $[P]$ 's resulting from the reaction of a constant  $B_0$  with different  $Po$ 's theoretically should vary only some ten times in titre.

Figs. 2, 3, 4 and 5 confirm this prediction and numerous other



experiments show the same general grouping of maximal  $[P]$  values. The destruction of  $P$  during lysis is sufficiently regular to maintain this relationship in the lysates after completion of bacteriophagy.

Fig. 16 also predicts the time for lysis with reasonable accuracy (compare with Fig. 11) and brings out an additional point, namely, that for a series of  $P$ - $B$  mixtures made with constant initial  $[B]$  and progressively larger  $P_0$ 's the maximal  $[P]$ 's will be in the reverse order of magnitude of the initial  $[P]$ 's. That is, the larger the  $P_0$  the smaller will be the maximal  $[P]$  reached during the reaction. Further, the regularity of  $P$  destruction during lysis should result in the lysates bearing the same titre relationships to one another as exist at the maxima when the reaction is completed. The experimental facts are in accord on both these points.

TABLE I

*Temperature Coefficients of P and B Formation and P and B Destruction*

|                    | Temperature <sub>1</sub> | Velocity constant $K_1$ | Temperature <sub>2</sub> | Velocity constant $K_2$ | $Q_{10}$ |
|--------------------|--------------------------|-------------------------|--------------------------|-------------------------|----------|
|                    | °C.                      |                         | °C.                      |                         |          |
| B growth.....      | 36                       | 0.6                     | 10                       | 0.006                   | 5.9      |
| P production.....  | 36                       | 1.6                     | 10                       | 0.06                    | 3.5      |
| B destruction..... | 36                       | 3.0                     | 10                       | 0.02                    | 6.9      |
| P ".....           | 36                       | 0.75                    | 10                       | 0.025                   | 3.7      |

*K. The Temperature Coefficients of P and B Formation and P and B Destruction.*—From the experiments already performed (Figs. 2, 3, 4, 5, 10 and 11) it is possible to get some idea of the temperature coefficients of the various processes included under "bacteriophagy." Table I lists the essential data.

These figures bring out again the fact that the  $P$ - $B$  system cannot be analyzed from the viewpoint of a single reaction. The temperature coefficients for  $P$  and  $B$  formation differ significantly and in very nearly the same ratio as those for  $P$  and  $B$  destruction differ.  $P$  formation has been shown to be conditioned by  $B$  reproduction but granting this intimate relationship, the difference in temperature coefficients of the two processes suggests that  $B$  growth and  $P$  formation are not one and the same reaction.  $P$  cannot be formed unless the  $B$  are

growing but once *B* growth has begun, and assuming that it continues, the reaction responsible for *P* production progresses as a concomitant process.

In just this same sense *P* inactivation during lytic destruction of *B* is conditioned upon *B* dissolution. The simplest theoretical case possible would be the building up of an unstable *P-B* system which would go to pieces *in toto*; then for every *B* lost, a certain number of P.U. would disappear. Experimentally this is true only in so far as the processes of *P* inactivation and *B* destruction are concomitant. However, that they are separate reactions is suggested by their different temperature coefficients.

#### DISCUSSION

The essential points in the reaction between 16 hour cultures of staphylococci and antistaphylococcus bacteriophage in nutrient broth of pH 7.6 at 36°C. and with mechanical shaking to maintain a uniform *B* suspension may be summarized as follows:

Bacterial growth in the *P-B* mixture does not differ from the growth of identical controls without *P* until such time as lysis begins, unless the initial *P/B* ratio is very high, an exception considered below. Both *B* growth and *P* formation exhibit a lag of approximately 0.5 hour. During this time *P* is rapidly diffusing into or becoming adsorbed to *B* at such a rate that by the end of the lag period only 10 to 30 per cent of the total *P* present is extracellular, the remainder being associated with the *B*. As the *B* growth curve enters the short phase of positive growth acceleration, equilibrium between the intracellular and extracellular *P* fractions becomes established and is maintained up to the time of lysis, extracellular [*P*] representing a small constant percentage of total [*P*]. This distribution of *P* on a constant percentage basis is suggestive of the manner in which a relatively simple chemical compound would be distributed and is not at all typical of the distribution one would expect if bacteriophage is a comparatively complex substance.

All experiments performed to investigate the relationship between *B* and *P* indicate that no *P* is produced without *B* growth, thus establishing the latter process as an essential conditioning factor for *P* formation. During the period of logarithmic *B* growth *P* formation

is likewise logarithmic but proceeds at a much faster rate. That is, the rate of  $P$  production is proportional to a power of the rate of  $B$  reproduction, making untenable the usual statement that each time a  $B$  divides a certain amount of  $P$  is formed.

When the value of  $\log P/B = 2.1$  ( $P/B = 125$ ) lysis occurs. This statement would be true, with different numerical values of course, for  $\log$  intracellular  $P/B$ ,  $\log$  extracellular  $P/B$  or, as written,  $\log$  total  $P/B$ . That is, there is no conclusive experimental evidence at this time as to which  $P$  fraction constitutes the essential conditioning agent for lysis. The most one can say is that lysis is not brought about by the attainment of a particular  $[P]$  per ml. but by a certain  $[P]$  per  $B$ . Obviously, this limiting value for any initial  $[B]$  is reached sooner the higher the initial  $[P]$ . When  $\log P/B$  at the time of mixing is already above 2.1 there is no growth of  $B$  and lysis soon occurs. The large value  $dP/dt$  as compared to that of  $dB/dt$  renders clear the experimental fact that the maximal  $[P]$ 's attained during the reaction of a constant  $[B]$  with various  $[P]$ 's fall within a very narrow range, those having been made with high initial  $[P]$  having a lower maximal  $[P]$  value than those made with low  $P_0$ .

The lytic destruction of  $B$  is again logarithmic with time just as are most death rate processes and the value  $-d \log B/dt$  for a particular initial  $[B]$  is constant for various  $P_0$ 's. There is good evidence that cells need not be growing in order to undergo lysis. As  $B$  are lysed a considerable percentage of the total maximal  $P$  formed is destroyed, the chief loss probably occurring in the intracellular fraction. The destruction of  $P$  is sufficiently regular to maintain the relationship established at the maxima. That is, lysates made by mixing a constant  $B_0$  with various  $P_0$ 's vary only some ten times in  $[P]$  after lysis is completed. Just as in the case of the maxima, the final  $[P]$ 's vary in inverse order of the  $P_0$ 's. The major portion (70 to 90 per cent) of the final  $P$  present after the completion of bacteriophagy is set free during the short period of bacterial dissolution.

There is a marked difference in the temperature coefficients of  $P$  and  $B$  formation. Further, the temperature coefficients of  $P$  and  $B$  destruction during lysis differ in almost the same ratio. These data suggest that while  $B$  growth may be an essential condition for  $P$  formation the two processes are basically separate reactions. A

similar interpretation would hold in the case of  $B$  dissolution and  $P$  inactivation.

Agitation of test series is a necessary condition in experiments of the sort described in this paper. Without mechanical shaking of the tubes  $B$  settle out. As an example of what may follow it is obvious that the critical  $P/B$  ratio for lysis can be reached in the relatively sparse  $B$  population of the supernatant and the  $[B]$  considerably depleted by lytic action, whereas if measures are taken to maintain a uniform  $[B]$  such a sequence does not occur. Anomalous results of this sort have been observed whenever shaking has been omitted.

### *The Kinetics of Bacteriophage Action*

(a) *The Fundamental Relationship between B Reproduction and P Formation during the Logarithmic Phase of B Growth.*—The following equation expresses the relationship between  $P$  formation and  $B$  growth during the logarithmic phase:

$$P/P_0 = (B/B_0)^n \quad (1)$$

where  $P$  = Phage/ml.  
and  $B$  = Bact./ml.

$$\therefore P = P_0 \frac{B^n}{B_0^n} \quad (2)$$

of which the differential form is:

$$dP/dB = P_0 \frac{n B^{n-1}}{B_0^n} \quad (3)$$

From (2)

$$P/B = P_0 \frac{B^{n-1}}{B_0^n} \quad (4)$$

The obvious step from this point is to solve Equation (8) below for  $B$  in terms of  $B_0$ ,  $B_c$  and  $t$ . Since it is experimentally true that lysis occurs when  $\log P/B = 2.1$ , substitution for  $B$  from Equation (8) in the right hand term of Equation (4) would make possible a direct solution for  $t_{(lysis)}$ . Practically, however, it is simpler to effect this

solution for  $t$  in two steps, first obtaining  $B$  (at lysis) from (4), substituting it in Equation (8) and solving the latter for  $t_{(\text{lysis})}$ .

(b) *Determination of Log B at Lysis.*—From Equation (4) it follows that:

$$\text{Log } (P/B) = \log P_0 + (n - 1) \log B - n \log B_0$$

At lysis  $\log P/B = 2.1$ .

$$\therefore \text{Log } B_{(\text{at lysis})} = \frac{2.1 + n \log B_0 - \log P_0}{n - 1} \quad (5)$$

(c) *General Relationship between  $P_0$  and  $t_{(\text{lysis})}$ .*—The plot of  $\log B$  against  $t$  is similar to the curve of an ordinary autocatalytic reaction. If it is assumed that:

$$dB/dt = gB(B_e - B) \quad (6)$$

where  $B_e$  is  $[B]$  at the maximum stationary growth phase and  $B$  the  $[B]$  at any time, it follows that:

$$\begin{aligned} dB/B(B_e - B) &= g dt \\ \therefore \int dB/B(B_e - B) &= \int g dt \end{aligned}$$

or

$$\begin{aligned} -\frac{1}{B_e} \log_e \frac{B_e - B}{B} &= gt + k \\ \therefore t &= -\frac{1}{gB_e} \log_e \frac{B_e - B}{B} - \frac{k}{g} \end{aligned} \quad (7)$$

*Evaluation of  $k$ :* When  $t = 0$ ,  $B = B_0$

$$\begin{aligned} \therefore -\frac{1}{gB_e} \log_e \frac{B_e - B_0}{B_0} - \frac{k}{g} &= 0 \\ \frac{k}{g} &= -\frac{1}{gB_e} \log_e \frac{B_e - B_0}{B_0} \end{aligned}$$

and

$$k = -\frac{1}{B_e} \log_e \frac{B_e - B_0}{B_0}$$

*Evaluation of  $gBe$ :* From equation (6)

$$dB/B \, dt = g (Be - B)$$

But

$$d \log_e B / dB = \frac{1}{B}$$

$$\therefore d \log_e B / dB \cdot dB / dt = g (Be - B)$$

Hence

$$d \log_e B / dt = g (Be - B)$$

When

$$B \ll Be, (Be - B) = Be$$

$$\therefore d \log_e B / dt = g Be$$

From Fig. 11, the plot of  $\log_{10} B$  against  $t$ ,  $d \log_{10} B / dt = 0.6$

$$\therefore gBe = (2.3) (0.6)$$

and

$$\frac{1}{gBe} = \frac{1}{(2.3) (0.6)}$$

Equation (7) then becomes:

$$\begin{aligned} t &= - \frac{2.3}{(2.3) (0.6)} \log_{10} \frac{Be - B}{B} + \frac{2.3}{(2.3) (0.6)} \log_{10} \frac{Be - B_0}{B_0} \\ &= 1.7 \left[ \log_{10} \frac{Be - B_0}{B_0} - \log_{10} \frac{Be - B}{B} \right] \\ &= 1.7 \left[ \log_{10} \frac{B (Be - B_0)}{B_0 (Be - B)} \right] \end{aligned} \quad (8)$$

Table II lists the values of  $B$  at lysis calculated from Equation (5) and the values for  $t_{(1/2) \text{ lysis}}$  obtained by substituting the calculated  $[B]$ 's in Equation (8).

The values of  $[B]$  at lysis and  $t$  for lysis calculated from the two equations compare well with the observed figures over a range of

initial  $[P]$  values varying 100,000 times. Below  $\log P_0 = 4.0$  agreement is not good for two reasons; first, because Equation (7) becomes extremely sensitive to the value assigned to  $B_e$  and second, because Equation (5) behaves similarly to values of  $n$ . However, Equation (7) predicts the experimental fact that with very low values of  $P_0$  lysis

TABLE II

$[B]$  at lysis, observed and calculated from

$$\log B = \frac{2.1 + n \log B_0 - \log P_0}{n - 1}$$

$t_{(\text{lysis})}$ , observed and calculated by substitution of the value of  $\log B$

$$\ln t = \frac{1}{g B_e} \log \frac{B (B_e - B_0)}{B_0 (B_e - B)}$$

$$\frac{1}{g B_e} = 1.7 \quad n = 4$$

| Log $P_0$ | Log $B_0$ | [B] at lysis |          | $t$ (-0.5) for lysis |          |
|-----------|-----------|--------------|----------|----------------------|----------|
|           |           | Calculated   | Observed | Calculated           | Observed |
| 9.0       | 7.4       | 7.6          | 7.6      | 0.30                 | 0.40     |
| 8.2       | 7.4       | 7.8          | 7.75     | 0.70                 | 0.70     |
| 7.75      | 7.4       | 8.0          | 7.90     | 1.04                 | 0.95     |
| 7.20      | 7.4       | 8.2          | 8.25     | 1.40                 | 1.45     |
| 7.0       | 7.4       | 8.25         | 8.30     | 1.50                 | 1.60     |
| 6.4       | 7.4       | 8.43         | 8.50     | 1.84                 | 1.90     |
| 6.0       | 7.4       | 8.6          | 8.65     | 2.16                 | 2.20     |
| 5.0       | 7.4       | 8.9          | 8.9      | 2.86                 | 2.80     |
| 4.85      | 7.4       | 8.95         | 8.95     | 3.00                 | 2.95     |
| 4.40      | 7.4       | 9.10         | 9.00     | 3.13                 | 3.10     |
| 4.00      | 7.4       | 9.20         | 9.10     | 3.98                 | 3.75     |

( $t - 0.5$  hr.) is used to allow for the lag of 0.5 hr. in  $B$  growth.

will never occur. That is, as  $B$  becomes large ( $B_e - B$ ) becomes progressively smaller until finally it has a value of zero. Solution for  $t$  is then indeterminate.

(d) *Relationship between Log  $P_0$  and  $T_{(\text{lysis})}$  for a Given Value of  $B_0$  during Logarithmic Growth Period.*—From Equation (5) at lysis

$$2.1 = \log P_0 + (n - 1) \log B - n \log B_0$$

During the logarithmic phase of  $B$  growth

$$B = B_0 e^{gt}$$

or

$$\log B = \log B_0 + (0.43) gt$$

Substituting this last value for  $\log B$  in (5):

$$2.1 = \log P_0 + (n - 1) (\log B_0 + 0.43 gt) - n \log B_0$$

or

$$\begin{aligned} t &= \frac{2.1 - (n - 1) \log B_0 - \log P_0 + n \log B_0}{(n - 1) (0.43) g} \\ &= \frac{9.5 - \log P_0}{1.7} \end{aligned} \quad (9)$$

Equation (9) applies numerically to the value  $\log B_0 = 7.4$

TABLE III

$t_{\text{lysis}}$  Observed and Calculated from Equation (9).

| Log $P_0$ | Calculated ( $t_{\text{lysis}} - 0.5$ )<br>$9.5 - \log P_0 / 1.7$ | Observed ( $t_{\text{lysis}} - 0.5$ ) |
|-----------|---|---------------------------------------|
| 7.0       | 1.47  | 1.5                                   |
| 6.0       | 2.06  | 2.2                                   |
| 5.0       | 2.65  | 2.7                                   |

( $t - 0.5$  hr.) is used to allow for the lag of 0.5 hr. in  $B$  growth.

Equation (9) predicts that with a constant initial  $B_0$  the plot of  $t_{\text{lysis}}$  against the  $\log P_0$  values used, will be a straight line. The kinetic analysis of the  $P$ - $B$  reaction thus rationalizes the method employed for quantitative  $P$  determinations (*q. v. i.* under "Methods") (2), a procedure developed originally on the basis of experimental observation alone. While Equation (9) deals with  $t$  values for the beginning of the lytic process and the  $t$  values utilized in the  $P$  determinations represent the time periods required to reduce the  $B$  suspension to a particular turbidity end-point, there is no discrepancy involved in applying the general relationship between  $P_0$  and  $t_{\text{lysis}}$  denoted in Equation (9) to the analytical method. Practically, the reduction of the heavy  $B$  suspension to a certain  $[B]$  end-point is much



to be preferred because the latter is technically convenient and decidedly more accurate than is the fixation of  $[B]$  at the beginning of lysis. The  $t$  values so obtained give just the same type of plot against  $\log Po$ 's as do the  $[B]$ 's at the start of lysis. That is, for any  $Po$  the time consumed in reducing  $[B]$  at the start of lysis to the  $[B]$  used as an end-point is practically constant within the range used for  $P$  determinations.

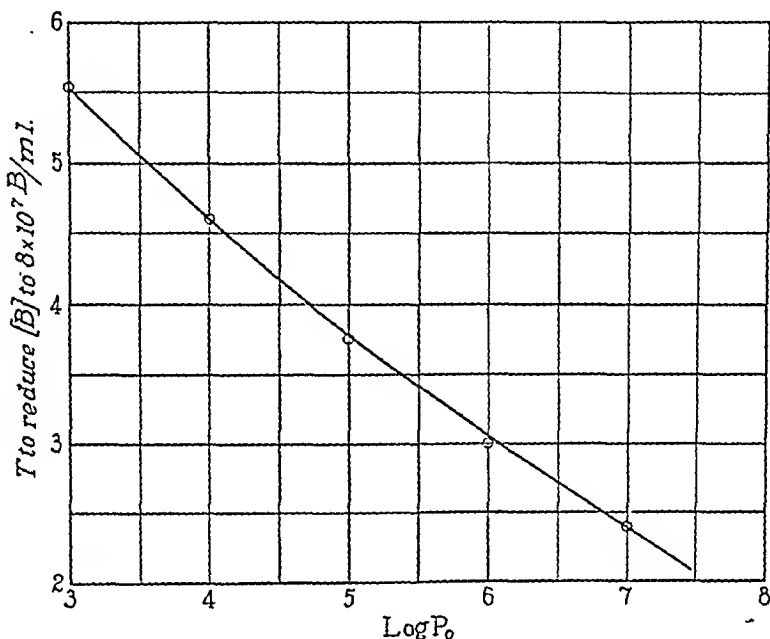


FIG. 17. Relationship between  $Po$  and  $t$  required to reduce  $[B]$  to a particular turbidity end-point. This curve is used in the method for determining  $P$  quantitatively.

It is also clear that theoretically the end-points given by  $\log Po$  values  $< 5.0$  should not lie on the standard plot of  $\log Po$  against  $t$  (a straight line), for  $B$  growth in such cases runs beyond the logarithmic phase into the phase of negative growth acceleration. Experimentally this is found to be true and in making  $P$  determinations the standard  $\log Po$  values used are 7.0, 6.0 and 5.0. The curve can of course be extended to  $\log Po = 4.0$  or even 3.0 but the points obtained will lie on a curve as indicated in Fig. 17. In the original description of the method for quantitative  $P$  determinations (2) the values of

standard log  $Po$ 's were 6.0, 5.0 and 4.0. The plot of these values against  $t$  (to reduce the  $[B]$  to a certain turbidity end-point) was shown as a straight line. Actually from log  $Po = 5.0$  on down it begins to curve slightly although practically no great difference is made in considering it a straight line for carrying out  $P$  determinations (cf. Fig. 17).

### Methods

*Method of Determining Bacteria.*—Routinely, cell concentrations were determined by comparing the unknown with turbidity standards. The latter were prepared each day from cultures of the staphylococcus being studied and readings were made under conditions giving a maximum Tyndall effect (1). In the range from  $3 \times 10^7$  B/ml. to  $15 \times 10^7$  B/ml. determinations can be made to within  $\pm 5$  per cent, a degree of accuracy entirely sufficient for present purposes. With higher  $[B]$ 's the unknowns were always diluted with broth so as to fall within these limits. The centrifuged sediment method (1) was employed regularly to check the turbidity estimates.

In the few instances requiring determination of living cell numbers the usual plate technic was followed. It will be noted in Fig. 1 that the living cell count as determined by plating is always slightly less than the total cell count as estimated by turbidity or centrifuged sediment measurements. This difference is probably due in part to the clumping together of cells, causing groups to appear as single colonies on the plate and to the fact that some cells of such preparations are not viable.

*Method of Determining Bacteriophage.*—The procedure employed is based upon the experimental observation that the time required to reduce a set concentration of growing phage-susceptible bacteria contained in a unit volume of  $P$ - $B$  mixture to an arbitrary turbidity end-point is a function of the amount of phage present at the start of the reaction. A detailed description of the method is given in a recent paper (2). The accuracy of phage determinations made by this procedure is  $\pm 3$  per cent in the range of standard  $P$  dilutions from  $10^{-3}$  to  $10^{-5}$  (corresponding to  $10^7$  to  $10^5$  P.U./ml.). In the present work  $P$  determinations were made on samples taken at various time intervals. Each unknown was at once diluted serially by tenths and 4 ml. aliquots of appropriate dilutions were mixed with 1 ml. portions of a freshly made staphylococcus suspension in broth containing  $12.5 \times 10^7$  B/ml.

The  $P$ - $B$  mixtures were placed in the water bath shaker at  $36^\circ\text{C}$ . Heavy growth occurred in all tubes except those made with  $P$  of very high titre. Such tubes were disregarded. Tubes containing from  $1 \times 10^5$  to  $1 \times 10^7$  P.U./ml. furnished at some time between 2 and 4 hours a sharp decrement in  $B$  for which the curve of  $[B]$  against time could be accurately fixed by turbidity readings at intervals of 0.1 hour when lysis was definitely under way and  $[B]$  was  $< 20 \times 10^7$ /ml. On the plot of  $[B]$  against time  $8 \times 10^7$  B/ml. was chosen as an end-point and the time

required for the lysing suspension to reach this value was read directly. Referring to the control plot of log P.U. against the time to reach the same end-point, the log P.U. corresponding to the time figure of the unknown was determined. Converting to antilogs and multiplying by the dilution gave the value of the unknown in P.U. This value was automatically checked in nearly all instances by the fact that two or three tubes would give readings in the range from  $1 \times 10^5$  to  $1 \times 10^7$  P.U./ml.

The staphylococcus suspensions used throughout a day's work were prepared from a single saline suspension made early in the day as described under "Set-up of kinetic experiments." The cell content of the latter was accurately known (centrifuged sediment method,  $\pm 2$  per cent), any required cell concentration could be made up readily, and control experiments showed no appreciable change in the numbers of bacteria present over a period of 8 hours. Likewise no qualitative alterations in the organisms were demonstrable. Thus, a control curve for *P* determinations made early in the day with *B* from a particular suspension could be matched closely with known *P* dilutions and the same *B* at any time during the day. This point is of importance since *P* estimations were made as much as 7 hours after the first control set was started. In order to rule out adequately any possible difficulty on this score two or three control sets of known [*P*]'s were run at various times during each series of *P* determinations.

*Set-Up of Kinetic Experiments.*—Unless otherwise noted all experiments were run as follows: A 16 hour culture of *S. aureus* grown in a Blake flask was suspended in saline and the mixture filtered through a Schleicher and Schüll Faltenfilter No. 588. The cell content of the suspension was determined by the centrifuged sediment method. Dilutions in broth were prepared and the bacteria immediately added to appropriate concentrations of phage contained in a series of tubes; controls without *P* were of course always included. Volume was kept constant at 5 ml. throughout. The tubes were placed in a shaker mounted in a water bath maintained at 36°C. At required time intervals tubes were removed for estimating the concentration of *B* and for making *P* determinations. After samples were pipetted off such tubes were discarded. Usually *P* determinations were made for total *P*/ml. and for extracellular *P*/ml. In the former case the contents of the tube were diluted by tenths in broth and 4 ml. aliquots of two or three serial dilutions were mixed with 1 ml. of a *B* suspension in broth made at this same time. The procedure outlined above was followed as to temperature, shaking, turbidity readings and calculations.

As a rule the *B* present in the unknown did not interfere with the *P* determination. This is quite what one would expect, for an average sample is diluted at least to  $10^{-2}$  or  $10^{-3}$  for analysis and the [*B*] required to yield barely visible turbidity is  $1 \times 10^7$  *B*/ml. Hence a suspension to interfere in this way must contain  $1 \times 10^{10}$  *B*/ml., a [*B*] four times the maximal value ordinarily encountered. The small residual [*B*]'s left in the dilutions usually used were negligible when compared to the  $2.5 \times 10^7$  *B*/ml. added for the *P* determination.

For the estimation of extracellular phage, a term employed merely to designate that portion of the total lytic agent not in or adsorbed to bacteria, the sample was centrifuged at 2600 R.P.M. for 0.1 hour immediately after removal from the bath. There is an obvious inaccuracy here since the separation of *B* from the supernatant is only partial at best. However, the number of *B* remaining in suspension was shown by control experiments to constitute a negligible percentage of the *B* thrown down and no appreciable error was introduced by considering the supernatant as *B*-free unless samples were allowed to stand a considerable length of time.

The separation of *B* and *P* is a gradual process and the reaction between them does not stop during the interval required for fairly complete sedimentation. Consequently, as a mean, the middle of the time interval required for centrifuging was used as the sampling time. Dilutions of the supernatant were run along with the total *P* determinations and in an identical manner.

#### SUMMARY AND CONCLUSIONS\*

The above data relating to the reaction between 16 hour cultures of *S. aureus* and antistaphylococcus bacteriophage in nutrient broth of pH 7.6 at 36°C. and with mechanical shaking to maintain a uniform *B* suspension, bring out the following points:

(a) *B* growth in *P*-*B* mixtures does not differ from growth in controls without *P* except in the case of a very high initial *P*/*B* ratio as noted below. There is no evidence that lytic destruction of *B* begins shortly after mixing *P* and *B* nor that *B* growth is stimulated by *P*, for the *B* growth curves in the presence of ordinary [*P*]'s and in controls are identical. Only at the sudden onset of the rapid lytic process does the *B* curve of a *P*-*B* mixture deviate from the control curve.

(b) *B* growth is an essential conditioning factor for *P* formation.

(c) Both *B* growth and *P* production exhibit short lags. During this time *P* diffuses into or becomes adsorbed to *B* so rapidly that by the end of the lag period only 10 to 30 per cent of the total *P* present is extracellular, the remainder being associated with the *B*.

(d) During the logarithmic *B* growth phase, *P* formation is also logarithmic but proceeds at a much faster rate. That is,  $dP/dt$  is

\* Symbols used:

*B* = Bacterium

*P* = Bacteriophage

[*B*] = Bacteria / ml.

[*P*] = Phage / ml.

*B*<sub>0</sub> = Initial value of [*B*]

*B*<sub>e</sub> = [*B*] at maximal stationary phase of  
*B* growth

*P*<sub>0</sub> = Initial value of [*P*]

proportional to a power of  $d B/d t$ . Consequently the statement that each time a  $B$  divides a certain amount of  $P$  is formed is not correct.

(e) As  $B$  growth enters the phase of positive acceleration equilibrium between the extracellular and intracellular  $P$  fractions becomes established and is maintained up to the onset of lysis, extracellular  $[P]$  representing a small constant percentage of total  $[P]$ . The distribution of  $P$  on a constant percentage basis suggests the manner in which a relatively simple chemical compound would be distributed and is not at all typical of the distribution one would expect if  $P$  were a complex organized parasite.

(f) When the value of  $\log P/B = 2.1$  lysis begins. Obviously, this limiting value for any initial  $[B]$  is reached sooner the higher the initial  $[P]$ . When  $\log P/B$  at the time of mixing  $P$  and  $B$  is already 2.1 or greater, there is no growth of  $B$  and lysis soon occurs.

(g) While there is good evidence that lysis is brought about by the attainment of a particular  $[P]$  per  $B$  and not by a certain  $[P]$  per ml., it is not clear at this time which of the ratios intracellular  $P/B$ , extracellular  $P/B$  or total  $P/B$  is the major conditioning factor for  $B$  lysis.

(h) Experimentally the maximal  $[P]$ 's of lysates made by mixing a constant initial  $[B]$  with widely varying  $Po$ 's fall within a relatively narrow range. This fact is explained by the large value of  $d \log P/d t$  as compared to  $d \log B/d t$ . That is, the loci of points at which  $\log P = 2.1 + \log B$  (maxima-lysis begins) on the curves of  $\log P$  against  $t$  originating in various  $[Po]$ 's will lie at a nearly constant level above the abscissa. Because of this same relationship the maximal  $[P]$ 's of such a series will be in the reverse order of magnitude of the  $Po$ 's, *i.e.*, the larger the  $Po$  the smaller will be the maximal  $[P]$  attained during the reaction (*cf.* Fig. 16).

(i) The lytic destruction of  $B$  is logarithmic with time, in this respect being similar to most death rate processes. The value  $-d \log B/d t$  for a particular initial  $[B]$  is constant for various initial values of  $[P]$ . There is good evidence that cells need not be growing in order to undergo lysis.

(j) During  $B$  lysis a considerable percentage of the total maximal  $P$  formed is destroyed, the chief loss probably occurring in the intracellular fraction. The major portion (70 to 90 per cent) of the final  $P$

present after the completion of bacteriophagy is set free during the brief phase of bacterial dissolution.

(k) When the entire process of bacteriophagy is completed the lysates are left with certain  $[P]$ 's determined by the foregone  $P$ - $B$  reaction. The destruction of  $P$  during lysis is sufficiently regular to maintain the relationship established at the maximal  $[P]$ 's. Therefore the final  $[P]$ 's have the same points in common that were noted in "h" as applying to the maximal  $[P]$ 's. That is, they all are grouped within a narrow range of  $[P]$  values, those having been made with high  $P_0$ 's being of lower titre than those made with low initial  $[P]$ 's.

(l) There is a significant difference in the temperature coefficients of  $P$  and  $B$  formation. Further, the temperature coefficients of  $P$  and  $B$  destruction during lysis differ in almost the same ratio. Consequently, while all experimental evidence postulates  $B$  growth as an essential conditioning factor for  $P$  formation, the temperature coefficient data suggest that the two processes are basically separate reactions. A similar interpretation holds in the case of  $B$  dissolution and  $P$  inactivation.

(m) The major events in the complete process of "bacteriophagy" are mathematically predictable. The  $[B]$  at which lysis occurs under certain standard conditions for given values of  $B_0$  and  $P_0$  may be calculated from the equation:

$$\text{Log } B = \frac{2.1 + n \log B_0 - \log P_0}{n - 1}$$

Substitution of this value for  $\log B$  in the equation:

$$t = \frac{1}{g B_0} \left[ \log \frac{B (B_0 - B_0)}{B_0 (B_0 - B)} \right]$$

gives satisfactory agreement with observed values for  $t_{(lysis)}$ .

(n) The kinetic analysis of the  $P$ - $B$  reaction predicts that the values of  $\log P_0$  plotted against  $t_{(lysis)}$  for a constant  $B_0$  will give a straight line. This plot is employed in a method for the quantitative estimation of  $P$  described in an earlier paper on the basis of experimental observation alone. Its use is made more rational by the facts given above.

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# ELECTROMETRIC DETERMINATIONS OF THE DISSOCIATION OF GLYCOCOLL AND SIMPLE PEPTIDES<sup>1</sup>

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Because of their importance in biochemistry and in the theory of amphoteric electrolytes, the acid and basic dissociation constants of amino acids and polypeptides have been measured by numerous investigators. A summary of available data has been published by Kirk and Schmidt (1). The agreement among the results of the several investigators is not very good. This is due to numerous causes, chief among them being the ever present uncertainty of the liquid junction potentials in the potentiometric studies, the difficulty of preparing compounds of sufficient purity and the neglect of certain corrections due to ionic activity.

The first two named difficulties are mechanical in nature. A method of obviating the last was undertaken by Simms (2) in a series of titration experiments wherein he employed the Brönsted and LaMer (3) limiting equation to obtain the activity coefficients of the ionic species involved. He worked with glycoll and studied its dissociation in the presence of NaCl and BaCl<sub>2</sub>. The further electrometric titration of glycoll and gelatin was conducted in the presence of certain antagonistic salt mixtures. The effect on the pH of the solution was complex.

The present paper reports the potentiometric determination of the apparent acidic and basic dissociation constants of glycoll and several peptides in aqueous solution. Measurements were also made in the presence of KCl or of K<sub>2</sub>SO<sub>4</sub> at equal ionic strength. Two methods, hydrolysis and titration, were employed. Hydrolysis afforded a basis of comparison of order of magnitude with the results

<sup>1</sup> This paper reports work done for a thesis submitted by Jesse P. Greenstein in partial fulfillment of the requirements for the degree of Ph.D. at Brown University.



of titration. This was desirable because uncertainties involved in determinations of hydrogen ion activities in a range far from the buffer region are clearly recognized. The hydrolysis method was suggested by the work of Denham (4) on inorganic salts. He pointed out that the potentiometric determination of the degree of hydrolysis constituted the most satisfactory method for this type of study, particularly in solutions of low hydrogen ion activity.

Winkelblech (5), on the other hand, employed the Bredig method of conductance to determine the degree of hydrolysis and studied several of the amino acids in this manner. But difficulties involved in the conductance method make it a rather unsatisfactory means for the study of the dissociation of these substances.

### *Materials and Apparatus*

The following peptides were synthesized according to the general methods of Emil Fischer: glycylglycocoll (6), alanylglycocoll (7), leucylglycocoll (8), methyl-leucylglycocoll (9), valylglycocoll (10), phenylalanylglycocoll (11) and glycylglycylglycocoll (12). All compounds were carefully boiled with absolute alcohol until no trace of chloride remained. They were then crystallized twice from pure distilled water in some cases under addition of alcohol and were dried at 100°C. Most of the intermediates were likewise crystallized from the appropriate solvents or else fractionally distilled. Previous to use in the experiments the peptides were kept for several hours over phosphorus pentoxide. No specific tests for purity could be made inasmuch as the compounds exhibit a wide range of fusion with decomposition within the fusion interval.

The usual scheme of measuring hydrogen electrode potentials was employed with a Type K potentiometer, a carefully calibrated Eppley standard cell and a reflecting type of galvanometer with lamp and scale. A saturated calomel electrode was used. All materials for its preparation were carefully purified. Its constant, determined by checking against a tenth normal calomel electrode, was 0.2454.

Hydrochloric acid was prepared by distilling a high grade commercial product and taking the middle fraction. It was standardized against carefully dried, pure sodium carbonate using methyl orange as indicator. Sodium hydroxide was prepared according to the method of Cornog (13) and was standardized against the standard hydrochloric acid. The KCl and  $K_2SO_4$  were from LaMotte and Kahlbaum stock respectively. The water was distilled from a Barnstead still and had a specific conductance of  $1 \times 10^{-6}$  mho. The salt bridge was the saturated KCl model recommended by Michaelis (14). This seems eminently satisfactory in comparison with other types in common use. The recent work of Guggenheim may be referred to in this connection (15).

All measurements were made in a thermostat at  $25^{\circ}\text{C.} \pm 0.1^{\circ}$ . Readings were taken until potentials checked to 0.2 mv. The value of  $\text{pK}_w$  at  $25^{\circ}\text{C.}$  was taken to be 13.895.

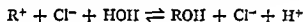
### Hydrolysis Studies

The method of hydrolysis involves, essentially, the potentiometric determination of the hydrogen ion activity ( $a_{\text{H}^+}$  or pH) in equimolar mixtures of the peptide and either HCl or NaOH. Lùden (16) has given a theoretical treatment of this type of reaction but his formulations were made some time before the advent of the modern theories of solution.

The following method of formulating the reactions involved was suggested by the work of Fenwick and Gilman (17) on the dissociation of the salts of certain complex organic compounds.

It is assumed that the acid reacts with the compound to form the hydrochloride whereas the base reacts to form the sodium salt. Complete dissociation of the salt in either case is taken for granted.

Letting R represent the general molecular grouping of the ampholyte, the reactions occurring in acid solution may be given thus:



$$\frac{a_{\text{R}^+} \times a_{\text{HOH}}}{a_{\text{ROH}} \times a_{\text{H}^+}} = \frac{k_b}{K_w} = \frac{a_{\text{R}^+}}{a_{\text{ROH}} \times a_{\text{H}^+}} = \frac{1}{K_a}$$

It is assumed that water possesses unit activity although Harned (18) in a series of calculations on the vapor pressure of water, showed that its activity at ordinary temperatures was slightly less than this value.

The degree of hydrolysis of the salt, represented by X, will be:

$$X = \frac{a_{\text{H}^+}}{\gamma_{\text{H}^+} \cdot C},$$

where C is the total concentration of ampholyte, reckoned on a molal basis, and  $\gamma_{\text{H}^+}$  is the activity coefficient of hydrogen ion concentration. Substituting in the mass action equation:

$$\frac{\lambda_{\text{R}^+} (1 - X)C}{\gamma_{\text{ROH}} \cdot XC a_{\text{H}^+}} = \frac{k_b}{K_w} = \frac{1}{K_a}$$

$$\frac{\gamma_{R^+} (\gamma_{H^+} C - a_{H^+})}{\gamma_{ROH} a_{H^+}^2} = \frac{1}{K_a}$$

Converting the expression into logarithmic form,

$$\log [\gamma_{H^+} C - a_{H^+}] + \log \gamma_{R^+} - \log \gamma_{ROH} + 2pH = pK_a$$

The values of the mean activity coefficient of hydrogen ion,  $\gamma_{H^+}$ , were taken from Schatchard's data (19) on solutions of HCl. It is assumed that, within the experimental error, the activity coefficient of hydrogen ion in the presence of ampholyte is approximately the same as in pure acid solution.

In order to determine the activity coefficient of the ampholyte ion, the ionic strength of the solution must be taken into account. Debye and Hückel (20), employing the assumption of complete dissociation of strong electrolytes, elaborated a theory which accounts for the deviations from the ideal state by taking into consideration the electrical forces among the ions. Their mathematical treatment was further simplified by Brönsted and LaMer (21) who found that the activity coefficient was related to the ionic strength,  $\mu$ , of the solution by the equation:  $-\log \gamma = Az^2\sqrt{\mu}$ , where  $A$  is a coefficient depending upon the dielectric constant of the medium and the absolute temperature and  $z$  refers to the valence. This equation is employed in the following formulation. The coefficient,  $A$ , at 25°C., is very nearly 0.5, so that,  $\log \gamma_{R^+} = -0.5z^2\sqrt{\mu} = -0.5\sqrt{C}$ , and assuming that the activity coefficient of the undissociated portion of a weak electrolyte is very nearly unity, the working equation is obtained:

$$\log [\gamma_{H^+} C - a_{H^+}] - 0.5 \sqrt{C} + 2pH - pK_a$$

A similar equation, based on reactions in alkaline solutions, is:

$$\log [\gamma_{OH^-} C - a_{OH^-}] - 0.5 \sqrt{C} + 2pOH = pK_b$$

Values of  $\gamma_{OH^-}$  are the mean values taken from Harned's data on NaOH solutions.

The following compounds were studied by the hydrolysis method: glycocoll, glycylglycocoll, alanylglycocoll, leucylglycocoll, methyl-leucylglycocoll, valylglycocoll, phenylalanylglycocoll, and glycylglycocoll.

The experimental procedure consisted in making up a normal solution of either HCl or NaOH in a volumetric flask and adding an equivalent normal weight of ampholyte. In some cases the initial solution was 0.1*N*, in others, particularly among the less soluble variety, the normality was lower. 50 cc. of the original solution were removed, transferred to another flask (100 cc.) and made up to the mark with distilled water. Several successive dilutions were made in this manner whereby the equivalence of peptide to reagent was kept constant and the concentration halved at each successive dilution. The pH of each dilution was determined at 25°C. and the dissociation constants calculated.

The potentials obtained in the case of phenylalanylglycocoll were erratic and considerable time elapsed before equilibrium was reached. Believing this to be an effect of the platinum black, we employed a quinhydrone electrode arranged according to Bülmann (22) in the hope of attaining equilibria more rapidly. This electrode yielded no better results, however, and it was discarded. Abderhalden and Suzuki (23) have shown that peptides containing aromatic radicals are rapidly split at the imide linkage in the presence of dilute acid and alkali even at ordinary temperatures.

The measurements by the hydrolysis method and the computed constants,  $pK_a$  and  $pK_b$ , are presented in Tables I and II. The results for glycocoll are less consistent than those for the peptides. This is probably due to impurity of the glycocoll.

A consideration of the deviations of the dissociation constants with variation in ionic strength, indicates in general a decided shift toward higher values of  $K_a$  and lower values of  $K_b$  as the ionic concentration is increased. These constants should give, if the formulations were adequate, a uniform value in the case of each compound. The mass action law has been stated in the requisite terms of activities; the failure to yield constants of more uniform character merits a critical analysis of the method of formulation.

It has been assumed that the activity coefficient of the ampholytic ion R, may be expressed in terms of the limiting law:

$$-\log \gamma_R = 0.5 z^2 \sqrt{\mu}$$

whereby the activity coefficient is solely a function of the valence and the ionic strength. Such a relationship will hold only in extremely dilute solutions ( $\mu < .01$ ) where the ionic diameters may be neglected. In the case of long ions, the fundamental equations of Debye and Hückel must be modified although as yet no theoretical treatment of the spatial distribution of charges has been attempted.

TABLE I

*Measurements by the Hydrolysis Method in Acid Solution*

| C     | $0.5\sqrt{C}$ | Glycocoll |         | Glycylglycocoll |        | Valylglycocoll |        | Alanylglycocoll |        |
|-------|---------------|-----------|---------|-----------------|--------|----------------|--------|-----------------|--------|
|       |               | pH        | $pK_a$  | pH              | $pK_a$ | pH             | $pK_a$ | pH              | $pK_a$ |
| 0.100 | 0.157         | 1.778     | 2.238   | 2.116           | 2.958  | .....          | .....  | 2.159           | 3.048  |
| 0.050 | 0.110         | 1.949     | 2.293   | 2.277           | 3.023  | .....          | .....  | 2.328           | 3.129  |
| 0.025 | 0.080         | 2.138     | (2.372) | 2.450           | 3.087  | 2.478          | 3.148  | 2.477           | 3.145  |
| 0.012 | 0.055         | 2.304     | 2.331   | 2.637           | 3.158  | 2.656          | 3.201  | 2.637           | 3.158  |
| 0.006 | 0.0385        | 2.490     | 2.322   | 2.785           | 3.134  | 2.810          | 3.193  | 2.795           | 3.154  |
| 0.003 | 0.0275        | 2.702     | 2.331   | 2.974           | 3.175  | 3.021          | 3.292  | 2.967           | 3.161  |

| C     | $0.5\sqrt{C}$ | Leucylglycocoll |        | Methylleucylglycocoll |        | Phenylalanylglycocoll |        | Glycylglycylglycocoll |        |
|-------|---------------|-----------------|--------|-----------------------|--------|-----------------------|--------|-----------------------|--------|
|       |               | pH              | $pK_a$ | pH                    | $pK_a$ | pH                    | $pK_a$ | pH                    | $pK_a$ |
| 0.100 | 0.157         | 2.165           | 3.060  | .....                 | .....  | .....                 | .....  | 2.191                 | 3.114  |
| 0.050 | 0.110         | 2.324           | 3.121  | 2.465                 | 3.398  | .....                 | .....  | 2.355                 | 3.186  |
| 0.025 | 0.080         | 2.467           | 3.123  | 2.587                 | 3.384  | 2.042                 | 2.118  | 2.524                 | 3.249  |
| 0.012 | 0.055         | 2.632           | 3.148  | 2.715                 | 3.334  | 2.204                 | 2.034  | 2.666                 | 3.226  |
| 0.006 | 0.0385        | 2.796           | 3.156  | 2.869                 | 3.332  | 2.416                 | 2.048  | 2.832                 | 3.248  |
| 0.003 | 0.0275        | 2.959           | 3.146  | 3.022                 | 3.294  | 2.648                 | 2.046  | 3.003                 | 3.256  |

TABLE II

*Measurements by the Hydrolysis Method in Basic Solution*

| C     | $0.5\sqrt{C}$ | Glycocoll |        | Glycylglycocoll |        | Valylglycocoll |        | Alanylglycocoll |        |
|-------|---------------|-----------|--------|-----------------|--------|----------------|--------|-----------------|--------|
|       |               | pOH       | $pK_b$ | pOH             | $pK_b$ | pOH            | $pK_b$ | pOH             | $pK_b$ |
| 0.050 | 0.110         | 2.879     | 4.293  | 3.687           | 5.920  | .....          | .....  | 3.870           | 6.287  |
| 0.025 | 0.080         | 3.019     | 4.309  | 3.824           | 5.933  | 4.048          | 6.383  | 3.933           | 6.157  |
| 0.012 | 0.055         | 3.159     | 4.296  | 3.987           | 5.976  | 4.175          | 6.355  | 4.071           | 6.147  |
| 0.006 | 0.0385        | 3.312     | 4.317  | 4.166           | 6.056  | 4.312          | 6.348  | 4.193           | 6.111  |
| 0.003 | 0.0275        | 3.432     | 4.251  | 4.301           | 6.036  | 4.479          | 6.393  | 4.323           | 6.081  |

| C     | $0.5\sqrt{C}$ | Leucylglycocoll |        | Methylleucylglycocoll |        | Phenylalanylglycocoll |        | Glycylglycylglycocoll |        |
|-------|---------------|-----------------|--------|-----------------------|--------|-----------------------|--------|-----------------------|--------|
|       |               | pOH             | $pK_b$ | pOH                   | $pK_b$ | pOH                   | $pK_b$ | pOH                   | $pK_b$ |
| 0.050 | 0.110         | 3.907           | 6.361  | 3.811                 | 6.169  | .....                 | .....  | 3.912                 | 6.371  |
| 0.025 | 0.080         | 3.975           | 6.235  | 3.895                 | 6.075  | 3.202                 | 4.680  | 4.026                 | 6.339  |
| 0.012 | 0.055         | 4.100           | 6.205  | 4.019                 | 6.043  | 3.542                 | 5.982  | 4.165                 | 6.335  |
| 0.006 | 0.0385        | 4.251           | 6.227  | 4.175                 | 6.075  | 3.894                 | 5.505  | 4.313                 | 6.351  |
| 0.003 | 0.0275        | 4.405           | 6.245  | 4.320                 | 6.075  | 4.215                 | 5.865  | 4.461                 | 6.357  |

Simms has suggested an empirical formula to apply to certain large organic ions. His equations however are confined to those concentrations where  $\mu$  varies from 0.01 to 0.1.

In the application of the fundamental equation, it has also been assumed that the peptide ion is univalent. Such a conception, in view of the several reactions described in the Introduction, seems somewhat doubtful. While the monovalent hydrochlorides of the peptides may be easily isolated, it is uncertain that such combinations are strictly stoichiometric. However, the question of extra valencies within the peptide molecule is so far from being settled that it need not be further considered here.

This leaves the problem of the ionic diameters to be considered. Probably this is where the present calculations are most in error. The simple limiting equation holds in concentrations where  $\gamma < 0.01$ . Above this limit, the activity coefficient of an ion must be a function of some value characteristic of all the ionic diameters in the solution. Where this factor is considered, the activity coefficient is defined by Hückel (24) as follows:

$$-\log \gamma = \frac{0.5 z^2 \sqrt{\mu}}{1 + 3.3 \times 10^7 r \sqrt{\mu}} + B(2\mu)$$

Here  $r$  may be interpreted as the average effective diameter of all the ions. Data relating to this variable are meager in the case of inorganic ions and are practically non-existent in the case of organic ions. The second term on the right hand side of the above equation represents the effect of the solute concentration on the dielectric constant. The zwitter ion with its dual charge and permanent moment might be expected to influence the dielectric constant considerably, and the experiments of Blüh (25) indicate that its value increases progressively with the concentration. Beyond the fact that at higher concentrations the dielectric constant of the solution is no longer the same as that of the pure solvent, little hope of arriving at the nature of the solute from capacity measurements can at present be held. It may be that the zwitter ion is an association complex in the sense of Bjerrum (26). Recent very accurate measurements (27) indicate that aqueous solutions of strong electrolytes slightly depress the dielectric constant of the solvent.

In view of the paucity of our knowledge concerning this problem, it seems best to leave the dissociation constants in their present form, referring to them as "apparent" constants since they are functions of several variables.

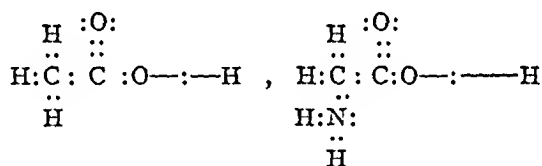
Turning our attention to the relative order of magnitude of the constants of the various compounds, we see a partial proof of the conceptions of Bjerrum and Adams.

Considering acid constants alone, their relative order of magnitude may be represented as follows:

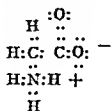
Phenylalanylglycocoll > glycocoll > glycylglycocoll > (alanyl-glycocoll, leucylglycocoll, valylglycocoll) > glycylglycylglycocoll > methylleucylglycocoll.

Bjerrum and Adams postulated a high degree of acidity for the glycocoll molecule in consequence of the presence of the highly electronegative group. The latter will further the dissociation of the hydrogen ion from the carboxyl group, such influence decreasing as the amino group is removed further from the carboxyl.

Representing the electronic structure of acetic acid and glycocoll thus:



it is apparent why glycocoll is a far stronger acid than acetic (some 100 times) and yet is neutral in pure water solution. The highly electronegative amino group will effect a deformation in the electronic nucleus, causing a shift in the position of the electron pairs about the carbon and oxygen atoms of the carboxyl group. This shift is toward the amino group. In the case of the oxygen-hydrogen linkage of the carboxyl group, the electron pair is drawn closer to the oxygen and farther away from the hydrogen. This amounts to a decrease in the strength of the bond, and an increase in the degree of dissociation of the acid. The proton thus set free combines immediately with the two unshared electrons of the nitrogen, forming the dipolar molecule, or zwitter ion.



It is apparent therefore that the farther away the amino group is from the carboxyl (the greater the number of carbon atoms intervening), the smaller will be the deformation of the electronic nucleus and the slighter the dissociation. Hence the observed decrease in the relative magnitude of the dissociation constants.

Two apparently significant exceptions occur to this rule, namely, in the case of phenylalanylglycocoll and of methyleucylglycocoll. In the former compound it is to be expected that the very strongly electronegative character of the phenyl group would be sufficient to make the compound a stronger acid than glycocoll. In the latter, introducing a methyl radical into the amino group decreases its electronegativity. This lowering of hydrogen-accepting capacity is apparently decreased as the dilution increases, and the compound forms an apparent exception to the rule of increased value of acid dissociation constant with increasing hydrogen ion activity.

In alkaline solution, the basic dissociation constants in general show a decided increase in value as the ionic strength decreases.

As a result of these particular studies, the generalization may be advanced that the dissociation constants are functions of the hydrogen ion activity and the ionic strength.

### *Titration Studies*

The following methods of formulating the reactions involved in titration measurements were suggested in part by the work of Simms and the discussions of Kolthoff (28), but several modifications have been introduced. If we consider the reaction in acid solution, we may formulate the equilibrium thus:



where  $^+\text{X}^-$  refers to the zwitter ion,  $\text{NH}_3^+\text{RCOO}^-$ . In consequence of the small ionic product of water, the concentration of ampholytic



anions in the presence of acid will be negligible. In mass action form, the equation is:

$$\frac{H^+ \times {}^+X^-}{H^+X} = K'_a$$

If  $\alpha$  represents the apparent "degree of dissociation" or the mol fraction of ampholytic cations ( $\alpha = \frac{H^+X}{C}$ , where  $C$  is the total concentration of ampholyte) then the mass action equation may be written thus:

$$K'_a = a_{H^+} \frac{(1 - \alpha)}{\alpha}$$

and in logarithmic form:

$$pK'_a = pH - \log \frac{(1 - \alpha)}{\alpha}$$

For electroneutrality in the solution the sum of the concentrations of cations must equal that of the anions or:

$$H^+ + H^+X = OH^- + Cl^-$$

and, assuming complete dissociation of HCl and negligible concentration of hydroxyl ions,

$$H^+ + H^+X = A$$

where  $A$  is the total concentration of acid.

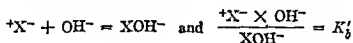
It follows that:

$$H^+ + \alpha C = A \text{ and } \alpha = \frac{A - H^+}{C}$$

but, inasmuch as  $\alpha$  is to be expressed as concentration, the  $H^+$  values, observed as activities, must be converted to concentrations by means of the equations:

$$H^+ = \frac{a_{H^+}}{\gamma_{H^+}} \text{ and } \log \gamma_{H^+} = -0.5 \sqrt{\mu}$$

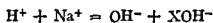
In basic solution, the reactions may be formulated thus:



Representing  $\alpha$  as the mol fraction of ampholytic anion,  $\alpha = \frac{XO^-}{C}$ ,

$$K'_b = a_{OH^-} \frac{(1 - \alpha)}{\alpha} \quad \text{and} \quad pK'_b = pOH - \log \frac{(1 - \alpha)}{\alpha}$$

For electroneutrality:



and assuming the sodium hydroxide, B, as completely dissociated:



whence

$$\alpha = \frac{B + H^+ - OH^-}{C}$$

Hydroxyl ion concentrations were calculated from the concentrations of hydrogen ions by means of the ionic product of water at 25°,  $K_w = 1.27 \times 10^{-14}$ .

The dissociation constants so calculated are of a hybrid or "apparent" character, inasmuch as the expression  $\log \frac{1 - \alpha}{\alpha}$  is expressed as concentration while pH is the negative logarithm of the hydrogen ion activity.

In order to obtain the "true" dissociation constants which shall be independent of the ionic strength of the solution, it is necessary to have an accurate knowledge of the activity coefficients of the ampholyte ions. Any attempt to derive a value for these coefficients on the basis of the Debye-Hückel theory involves certain *a priori* assumptions which have been considered in the previous section on hydrolysis.

The experimental technique consisted in adding acid or base to a constant quantity of ampholyte alone in solution, and in the presence of salt. A 0.002 N solution of either HCl or NaOH was made up in a 200 cc. volumetric flask. To

this was added a weighed amount of ampholyte so that its concentration would be 0.02 M. 25 cc. samples were placed in 50 cc. volumetric flasks with the desired amounts of 0.1 N HCl or NaOH, and with 5 cc. of 0.5 M KCl or 0.166 M  $K_2SO_4$ , or with no salt. The aliquots were then made up to the mark with distilled water. The ionic strength, then, of each of the salts was the same (0.05M). The final concentration of total ampholyte in each sample was 0.01 M.

Potentiometric data were obtained on the following compounds: glycoll, glycyglycoll, alanyglycoll, leucylglycoll, methylleucylglycoll, and glycyglycyglycoll. The dissociation constants of each ampholyte were calculated by means of the above formulae. The results are reported in Tables III-VIII. The lack of a sufficient supply of glycyglycyglycoll was the cause of the paucity of data on this compound.

TABLE III

*Electrometric Titration of Glycoll (0.01 M)  
With HCl*

| A     | HCl without salt |                  | HCl with KCl = 0.05M |                  | HCl with $K_2SO_4$ = 0.05M |                  |
|-------|------------------|------------------|----------------------|------------------|----------------------------|------------------|
|       | pH               | pK' <sub>a</sub> | pH                   | pK' <sub>a</sub> | pH                         | pK' <sub>a</sub> |
| 0.001 | 3.419            | 2.232            | 3.495                | 2.292            | 3.614                      | 2.484            |
| 0.002 | 3.123            | 2.262            | 3.182                | 2.295            | 3.279                      | 2.461            |
| 0.003 | 2.973            | 2.335            | 2.979                | 2.271            | 3.093                      | 2.480            |
| 0.004 | 2.818            | 2.311            | 2.849                | 2.289            | 2.962                      | 2.506            |
| 0.005 | 2.707            | 2.314            | 2.725                | 2.257            | 2.852                      | 2.516            |
| 0.006 | 2.612            | 2.311            | 2.629                | 2.244            | 2.744                      | 2.502            |
| 0.007 | 2.511            | 2.264            | 2.539                | 2.209            | 2.668                      | 2.526            |
| 0.008 | 2.450            | 2.287            | 2.467                | 2.200            | 2.597                      | 2.540            |

*Titration with NaOH*

| B     | NaOH without salt |                  | NaOH with KCl = 0.05M |                  | NaOH with $K_2SO_4$ = 0.05M |                  |
|-------|-------------------|------------------|-----------------------|------------------|-----------------------------|------------------|
|       | pH                | pK' <sub>b</sub> | pH                    | pK' <sub>b</sub> | pH                          | pK' <sub>b</sub> |
| 0.001 | 8.715             | 4.226            | 8.656                 | 4.285            | 8.697                       | 4.244            |
| 0.002 | 9.116             | 4.174            | 9.033                 | 4.257            | 9.059                       | 4.231            |
| 0.003 | 9.319             | 4.204            | 9.262                 | 4.271            | 9.267                       | 4.256            |
| 0.004 | 9.526             | 4.185            | 9.456                 | 4.257            | 9.462                       | 4.251            |
| 0.005 | 9.678             | 4.208            | 9.621                 | 4.267            | 9.631                       | 4.257            |
| 0.006 | 9.842             | 4.215            | 9.773                 | 4.289            | 9.792                       | 4.268            |
| 0.007 | 10.003            | 4.238            | 9.942                 | 4.305            | 9.974                       | 4.271            |
| 0.008 | 10.184            | 4.268            | 10.133                | 4.330            | 10.135                      | 4.328            |

TABLE IV  
*Electrometric Titration of Glycylglycocol (0.01 M)*  
*With HCl*

| A     | HCl without salt |                  | HCl with KCl = 0.05μ |                  | HCl with K <sub>2</sub> SO <sub>4</sub> = 0.05μ |                  |
|-------|------------------|------------------|----------------------|------------------|---|------------------|
|       | pH               | pK' <sub>a</sub> | pH                   | pK' <sub>a</sub> | pH  | pK' <sub>a</sub> |
| 0.001 | 4.110            | 3.115            | 4.145                | 3.146            | 4.128   | 3.129            |
| 0.002 | 3.790            | 3.140            | 3.790                | 3.128            | 3.829   | 3.173            |
| 0.003 | 3.541            | 3.109            | 3.578                | 3.137            | 3.622   | 3.188            |
| 0.004 | 3.362            | 3.101            | 3.392                | 3.119            | 3.458   | 3.199            |
| 0.005 | 3.218            | 3.104            | 3.248                | 3.119            | 3.309   | 3.197            |
| 0.006 | 3.077            | 3.092            | 3.108                | 3.100            | 3.179   | 3.203            |
| 0.007 | 2.944            | 3.075            | 2.969                | 3.084            | 3.049   | 3.194            |
| 0.008 | 2.810            | 3.041            | 2.849                | 3.049            | 2.945   | 3.216            |

*Titration with NaOH*

| B     | NaOH without salt |                  | NaOH with KCl = 0.05μ |                  | NaOH with K <sub>2</sub> SO <sub>4</sub> = 0.05μ |                  |
|-------|-------------------|------------------|-----------------------|------------------|--|------------------|
|       | pH                | pK' <sub>b</sub> | pH                    | pK' <sub>b</sub> | pH   | pK' <sub>b</sub> |
| 0.001 | 7.304             | 5.637            | 7.047                 | (5.894)          | 7.267  | 5.674            |
| 0.002 | 7.676             | 5.617            | 7.590                 | 5.703            | 7.607  | 5.686            |
| 0.003 | 7.854             | 5.673            | 7.792                 | 5.735            | 7.818  | 5.709            |
| 0.004 | 8.059             | 5.660            | 7.993                 | 5.726            | 8.003  | 5.716            |
| 0.005 | 8.225             | 5.670            | 8.162                 | 5.733            | 8.169  | 5.726            |
| 0.006 | 8.387             | 5.684            | 8.338                 | 5.733            | 8.356  | 5.715            |
| 0.007 | 8.594             | 5.669            | 8.533                 | 5.730            | 8.539  | 5.724            |
| 0.008 | 8.803             | 5.694            | 8.758                 | 5.739            | 8.769  | 5.728            |

TABLE V  
*Electrometric Titration of Alanylglycocoll (0.01 M)*  
*With HCl*

| A     | HCl without salt |                  | HCl with KCl = 0.05 $\mu$ |                  | HCl with K <sub>2</sub> SO <sub>4</sub> = 0.05 $\mu$ |                  |
|-------|------------------|------------------|---------------------------|------------------|--|------------------|
|       | pH               | pK' <sub>a</sub> | pH                        | pK' <sub>a</sub> | pH   | pK' <sub>a</sub> |
| 0.001 | .....            | .....            | 4.164                     | 3.169            | 4.206  | 3.211            |
| 0.002 | 3.773            | 3.123            | 3.813                     | 3.155            | 3.857  | 3.205            |
| 0.003 | 3.571            | 3.143            | 3.610                     | 3.176            | 3.649  | 3.219            |
| 0.004 | 3.399            | 3.143            | 3.423                     | 3.156            | 3.473  | 3.217            |
| 0.005 | 3.248            | 3.141            | 3.275                     | 3.154            | 3.326  | 3.220            |
| 0.006 | 3.106            | 3.132            | 3.126                     | 3.129            | 3.204  | 3.237            |
| 0.007 | 2.967            | 3.110            | 2.976                     | 3.084            | 3.064  | 3.217            |
| 0.008 | 2.839            | 3.085            | 2.866                     | 3.058            | 2.964  | 3.246            |

*Titration with NaOH*

| B     | NaOH without salt |                  | NaOH with KCl = 0.05 $\mu$ |                  | NaOH with K <sub>2</sub> SO <sub>4</sub> = 0.05 $\mu$ |                  |
|-------|-------------------|------------------|----------------------------|------------------|---|------------------|
|       | pH                | pK' <sub>b</sub> | pH                         | pK' <sub>b</sub> | pH  | pK' <sub>b</sub> |
| 0.001 | 7.331             | 5.610            | 7.260                      | 5.681            | 7.277   | 5.664            |
| 0.002 | 7.688             | 5.605            | 7.619                      | 5.674            | 7.629   | 5.664            |
| 0.003 | 7.886             | 5.641            | 7.839                      | 5.688            | 7.844   | 5.683            |
| 0.004 | 8.071             | 5.648            | 8.011                      | 5.708            | 8.033   | 5.686            |
| 0.005 | 8.248             | 5.647            | 8.186                      | 5.709            | 8.211   | 5.684            |
| 0.006 | 8.423             | 5.648            | 8.377                      | 5.694            | 8.380   | 5.691            |
| 0.007 | 8.631             | 5.632            | 8.570                      | 5.693            | 8.588   | 5.675            |
| 0.008 | 8.837             | 5.660            | 8.778                      | 5.719            | 8.792   | 5.705            |

TABLE VI  
*Electrometric Titration of Leucylglycocoll (0.01 M)*  
*With HCl*

| A     | HCl without salt |                  | HCl with KCl = 0.05M |                  | HCl with K <sub>2</sub> SO <sub>4</sub> = 0.05M |                  |
|-------|------------------|------------------|----------------------|------------------|---|------------------|
|       | pH               | pK' <sub>a</sub> | pH                   | pK' <sub>a</sub> | pH  | pK' <sub>a</sub> |
| 0.001 | 4.152            | 3.163            | 4.176                | 3.181            | 4.194   | 3.199            |
| 0.002 | 3.798            | 3.151            | 3.808                | 3.151            | 3.849   | 3.197            |
| 0.003 | 3.577            | 3.149            | 3.609                | 3.173            | 3.649   | 3.219            |
| 0.004 | 3.392            | 3.136            | 3.434                | 3.171            | 3.477   | 3.221            |
| 0.005 | 3.252            | 3.147            | 3.275                | 3.154            | 3.328   | 3.222            |
| 0.006 | 3.103            | 3.127            | 3.132                | 3.139            | 3.191   | 3.219            |
| 0.007 | 2.978            | 3.127            | 2.988                | 3.103            | 3.072   | 3.230            |
| 0.008 | 2.840            | 3.090            | 2.874                | 3.094            | 2.969   | 3.255            |

*Titration with NaOH*

| B     | NaOH without salt |                  | NaOH with KCl = 0.05M |                  | NaOH with K <sub>2</sub> SO <sub>4</sub> = 0.05M |                  |
|-------|-------------------|------------------|-----------------------|------------------|--|------------------|
|       | pH                | pK' <sub>b</sub> | pH                    | pK' <sub>b</sub> | pH   | pK' <sub>b</sub> |
| 0.001 | 7.209             | 5.733            | 7.154                 | 5.787            | 7.164  | 5.777            |
| 0.002 | 7.577             | 5.716            | 7.499                 | 5.794            | 7.506  | 5.787            |
| 0.003 | 7.768             | 5.759            | 7.709                 | 5.818            | 7.712  | 5.815            |
| 0.004 | 7.968             | 5.751            | 7.905                 | 5.814            | 7.910  | 5.809            |
| 0.005 | 8.133             | 5.762            | 8.081                 | 5.814            | 8.071  | 5.824            |
| 0.006 | 8.321             | 5.750            | 8.253                 | 5.818            | 8.260  | 5.811            |
| 0.007 | 8.511             | 5.752            | 8.448                 | 5.815            | 8.451  | 5.812            |
| 0.008 | 8.725             | 5.772            | 8.653                 | 5.844            | 8.663  | 5.834            |

TABLE VII  
*Electrometric Titration of Methylleucylglycine (0.01 M)  
 With HCl*

| A     | HCl without salt |                  | HCl with KCl = 0.05μ |                  | HCl with K <sub>2</sub> SO <sub>4</sub> = 0.05μ |                  |
|-------|------------------|------------------|----------------------|------------------|---|------------------|
|       | pH               | pK' <sub>a</sub> | pH                   | pK' <sub>a</sub> | pH  | pK' <sub>a</sub> |
| 0.001 | 4.216            | 3.232            | 4.226                | 3.237            | 4.184   | 3.189            |
| 0.002 | 3.846            | 3.205            | 3.890                | 3.243            | 3.913   | 3.269            |
| 0.003 | 3.631            | 3.212            | 3.665                | 3.237            | 3.698   | 3.275            |
| 0.004 | 3.453            | 3.208            | 3.490                | 3.236            | 3.521   | 3.273            |
| 0.005 | 3.304            | 3.212            | 3.331                | 3.225            | 3.377   | 3.281            |
| 0.006 | 3.154            | 3.196            | 3.184                | 3.210            | 3.233   | 3.275            |
| 0.007 | 3.011            | 3.175            | 3.040                | 3.182            | 3.109   | 3.283            |
| 0.008 | 2.879            | 3.155            | 2.912                | 3.162            | 2.996   | 3.298            |

*Titration with NaOH*

| B     | NaOH without salt |                  | NaOH with KCl = 0.05μ |                  | NaOH with K <sub>2</sub> SO <sub>4</sub> = 0.05μ |                  |
|-------|-------------------|------------------|-----------------------|------------------|--|------------------|
|       | pH                | pK' <sub>b</sub> | pH                    | pK' <sub>b</sub> | pH   | pK' <sub>b</sub> |
| 0.001 | 7.340             | 5.601            | 7.289                 | 5.652            | .....  | .....            |
| 0.002 | 7.680             | 5.613            | 7.636                 | 5.657            | 7.648  | 5.645            |
| 0.003 | 7.891             | 5.636            | 7.849                 | 5.678            | 7.851  | 5.676            |
| 0.004 | 8.089             | 5.630            | 8.032                 | 5.687            | 8.037  | 5.682            |
| 0.005 | 8.257             | 5.638            | 8.208                 | 5.687            | 8.213  | 5.682            |
| 0.006 | 8.434             | 5.637            | 8.396                 | 5.675            | 8.387  | 5.684            |
| 0.007 | 8.629             | 5.634            | 8.575                 | 5.688            | 8.587  | 5.676            |
| 0.008 | 8.840             | 5.657            | 8.797                 | 5.700            | 8.800  | 5.697            |

TABLE VIII  
*Electrometric Titration of Glycylglycylglycocol (0.01 M)*  
*With HCl*

| A     | HCl without salt |                  | HCl with KCl = 0.05M |                  |
|-------|------------------|------------------|----------------------|------------------|
|       | pH               | pK' <sub>a</sub> | pH                   | pK' <sub>a</sub> |
| 0.001 | 4.209            | 3.225            | 4.236                | 3.247            |
| 0.003 | 3.648            | 3.231            | 3.671                | 3.246            |
| 0.005 | 3.318            | 3.229            | 3.353                | 3.252            |
| 0.007 | 3.032            | 3.204            | 3.052                | 3.199            |

*Titration with NaOH*

| B     | NaOH without salt |                  | NaOH with KCl = 0.05M |                  |
|-------|-------------------|------------------|-----------------------|------------------|
|       | pH                | pK' <sub>b</sub> | pH                    | pK' <sub>b</sub> |
| 0.001 | 7.003             | (5.938)          | 7.032                 | 5.909            |
| 0.003 | 7.656             | 5.871            | 7.615                 | 5.912            |
| 0.005 | 8.035             | 5.860            | 7.978                 | 5.917            |
| 0.007 | 8.390             | 5.873            | 8.372                 | (5.891)          |



## DISCUSSION

On an examination of the data, the following facts are brought to light. The first is that in general the dissociation constants are a function of the hydrogen ion activity. They show definite trends: the values of  $K_a$  and  $K_b$  of each compound increasing with increasing hydrogen ion activity in solution without the presence of salt and in the presence of KCl; while  $K_a$  in the presence of  $K_2SO_4$  decreases with hydrogen ion activity whereas  $K_b$  in the presence of  $K_2SO_4$  increases. At equal ionic strengths the acid constants in the presence of KCl are greater than in the presence of  $K_2SO_4$ , the reverse holding true of the basic constants in alkaline solution.

The following chart indicates the trend of the apparent dissociation constants in each titration series with  $H^+$  ion activity and ionic strength: the arrows pointing upwards indicate increasing values, those downward, decreasing values.

|                |                   |                    |                  |
|----------------|-------------------|--------------------|------------------|
| Without salt   | $K'_a \uparrow$   | $a_{H^+} \uparrow$ | $\mu \uparrow$   |
|                | $\vee$            | $\vee$             | $\wedge$         |
| With KCl       | $K'_a \uparrow$   | $a_{H^+} \uparrow$ | $\mu \uparrow$   |
|                | $\vee$            | $\vee$             | $\parallel$      |
| With $K_2SO_4$ | $K'_a \downarrow$ | $a_{H^+} \uparrow$ | $\mu \uparrow$   |
|                |                   |                    |                  |
| Without salt   | $K'_b \uparrow$   | $a_{H^+} \uparrow$ | $\mu \downarrow$ |
|                | $\vee$            | $\wedge$           | $\wedge$         |
| With $K_2SO_4$ | $K'_b \uparrow$   | $a_{H^+} \uparrow$ | $\mu \downarrow$ |
|                | $\vee$            | $\wedge$           | $\parallel$      |
| With KCl       | $K'_b \uparrow$   | $a_{H^+} \uparrow$ | $\mu \downarrow$ |

A satisfactory explanation of the deviations of these constants is difficult to give on purely theoretical grounds. It is known that the apparent dissociation constant of a weak electrolyte is markedly dependent upon the ionic strength of the solution, and in the case of the ampholyte should decrease in absolute value on the addition of other electrolytes.

Stated in terms of the simplified Debye-Hückel theory:

$$-\log \gamma = 0.5 z^2 \sqrt{\mu},$$

if the equilibrium of the ampholyte be represented as above,

$$K'_a = c_{H^+} \frac{(1 - \alpha)}{\alpha}$$

where  $K'_a$  is the apparent dissociation constant, then the true dissociation constant based on activities will be  $K_a = K'_a \frac{1}{\gamma}$ , assuming that the activity coefficient of the undissociated ampholyte is equal to unity.

Therefore

$$\log K'_a = \log K_a - 0.5 z^2 \sqrt{\mu}$$

Inasmuch as  $K_a$  is a constant, the apparent dissociation constant should decrease as the ionic strength increases. The same relation should hold for  $K'_b$ .

On the contrary, in acid solution in each titration series with the exception of those involving  $K_2SO_4$ , we observe an increase of the apparent constant with increasing ionic strength. Furthermore, according to the principle of ionic strength, the constants in the presence of the salts should be equal at equal ionic strengths. It must be remembered, however, that this law is of limited validity, holding only in such very dilute solutions that specific ion effects are negligible. In the present studies, the difference in the specific effects of the sulfate and chloride ions is clearly evidenced. The work of Harned (29) on the activity coefficients of sulfuric acid in the presence of sulfate ion is illuminating in this regard. He found a decreased activity of the acid in the presence of this ion as the latter's concentration increased. It seems probable that the decrease is due to the formation of  $HSO_4$  ions. Kraus (30), listing the activity coefficients of  $K_2SO_4$  solutions, indicated the very low activity coefficient of this salt as compared with that of  $KCl$ . The latter, for example, at 0.01 M has a value of 0.922, the former at the same concentration a value of 0.687. Sulfuric acid yields a coefficient of 0.617. The effect of the sulfate ion will be to lower the ionic activity of the medium; increase in ionic strength will then so favor the formation of ampholyte ions that their excess will outweigh the effect of reduced hydrogen ion activity on the magnitude of the apparent constant. This is indicated by a comparison of  $\alpha$  values and  $H^+$  ion activities in acid-ampholyte,  $KCl$ -ampholyte, and  $K_2SO_4$ -ampholyte solutions:

$$\alpha_{K_2SO_4} > \alpha_{no\ salt} > \alpha_{KCl}$$

$$a_{H^+ no\ salt} > a_{H^+ KCl} > a_{H^+ K_2SO_4}$$

It may be that the lowering of the degree of dissociation in the presence of KCl is due to a common ion effect. The apparent disagreement of  $K'_a$  with the Debye-Hückel equation confirms the work of Simms on glycocoll.

The agreement of the equation with theory in the case of the apparent basic constants is substantially good. Here the constants in general decrease as the ionic strength is increased.

It would be unwise to speculate further on specific ion effects in view of the slight amount of data now available in the literature. The present paper is simply a contribution to the study of ampholytes in salt solutions, and a more nearly complete elucidation must await further theoretical advances.

#### SUMMARY

1. The apparent acid and basic dissociation constants were determined potentiometrically by the methods of hydrolysis and of titration for the following ampholytes: Glycocoll, glycylglycocoll, alanyl-glycocoll, valylglycocoll, leucylglycocoll, methylleucylglycocoll, phenylalanyl-glycocoll and glycylglycylglycocoll. The constants were also determined in the presence of KCl and of  $K_2SO_4$  at equal ionic strength.

2. In general, the relative order of magnitude of the constants decreased as the number of carbon atoms between amino and carboxyl groups increased. An explanation of this is offered on the basis of theories of electronic structure.

3. The application of the modern concepts of solutions to the case of the ampholytic ions is discussed. The inadequacy of the present theories is pointed out.

4. The constants were found, in general, to be functions of the hydrogen ion activity and the ionic strength of the solutions. Apparent contradictions to the Debye-Hückel theory are pointed out and partially explained on the basis of specific ion effects.

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# THE KINETICS OF PENETRATION

## III. EQUATIONS FOR THE EXCHANGE OF IONS

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The assumption is frequently made that an exchange of ions occurs in living cells. Since thermodynamics affords no test of this assumption the problem must be attacked kinetically and a mathematical treatment is therefore necessary. The brief outline given here is a preliminary attempt in this direction.

In the subsequent treatment we shall for convenience assume that the activity coefficients equal unity, that the electrolytes are monovalent, that when the concentrations of electrolytes are not the same within and without the osmotic pressures are rendered equal by the presence of non-electrolytes,<sup>1</sup> and that conditions are such that if a non-electrolyte penetrates, the time curve is of the first order. It is also convenient to assume that the solutions are not buffered so that when  $H^+$  enters it appears as such inside.<sup>2</sup>

Supposing an exchange of cations, the protoplasm being impermeable to anions, it is evident that, other things being equal, the rate of exchange will be proportional to the activities (here equivalent to concentrations). For example, if we have chiefly  $H^+$  outside and  $K^+$  inside the rate at which  $H^+$  passes inward in exchange for  $K^+$  will be proportional to  $H_o K_i$ , where  $H_o$  is the external concentration of  $H^+$  and  $K_i$  is the internal concentration of  $K^+$ . The outward passage of  $H^+$  (in exchange for  $K^+$ ) will be proportional to  $H_i K_o$ , so that the net rate will be proportional to the difference between the two, or to  $H_o K_i - H_i K_o$ .

<sup>1</sup> In case a divalent cation is exchanged for 2 monovalent cations there will be a change in osmotic pressure and a corresponding movement of water.

<sup>2</sup> In case the solutions are buffered we can put  $H_i$  equal to the titratable acidity in place of the concentration of  $H^+$ .

Let us now consider conditions at equilibrium.<sup>3</sup> Since all the anions are indiffusible we shall have a special kind of Donnan equilibrium with the relation (quite the opposite of that where the anions are diffusible)

$$\frac{A_o}{A_i} = \frac{C_o}{C_i}$$

where  $A$  is the total concentration of (monovalent) anions and  $C$  is the total concentration of (monovalent) cations. If the anions consist of several species  $A_1, A_2$ , etc., we shall have

$$\frac{A_{1o}}{A_{1i}} = \frac{A_{2o}}{A_{2i}} = \frac{A_o}{A_i}$$

and in the same way

$$\frac{C_{1o}}{C_{1i}} = \frac{C_{2o}}{C_{2i}} = \frac{C_o}{C_i} = \frac{A_o}{A_i}$$

Presumably the rate of exchange, other things being equal, will depend on the slower ion. Assuming that  $K^+$  moves more slowly through the protoplasm<sup>4</sup> than  $H^+$  we may say that since  $H^+$  cannot pass through the protoplasm unless  $K^+$  passes through at the same time in the opposite direction we need only consider the rate of movement of  $K^+$ . We may define the permeability to  $K^+$ , which we may call  $P_K$ , as the amount of  $K^+$  passing through the protoplasm into the vacuole in unit time under standard conditions, *i.e.*, when the protoplasm has unit surface and when the value of  $H_oK_i - H_iK_o$  equals unity.<sup>5</sup>

We may then put<sup>6</sup> for the net amounts passing through

$$- \frac{dK_i}{dt} = \frac{dH_i}{dt} = P_K [(H_o)(K_i) - (H_i)(K_o)]$$

<sup>3</sup> Cf. Netter, H., *Arch. ges. Physiol.*, 1928, 220, 107.

<sup>4</sup> The movement through the entire protoplasm (consisting of both aqueous and non-aqueous layers) is here referred to: it may be complicated by chemical combination, adsorption, etc.

<sup>5</sup> Cf. Osterhout, W. J. V., *J. Gen. Physiol.*, 1929-30, 13, 261.

<sup>6</sup> Here

$$- \frac{dK_i}{dt} = \frac{dK_o}{dt}$$

and as the solutions are not buffered

$$\frac{dH_i}{dt} = - \frac{dH_o}{dt}$$

If the amount of  $K_i$  at the beginning be called  $K_{ib}$  we may say that (since  $K^+$  must go out when  $H^+$  comes in)  $K_i$  must equal  $K_{ib}$  less the net amount of  $H^+$  which has entered, which is  $H_i - H_{ib}$  (where  $H_{ib}$  denotes the amount of  $H_i$  at the beginning). Hence we have

$$K_i = K_{ib} - (H_i - H_{ib})$$

Substituting this value we have<sup>7</sup>

$$\frac{dH_i}{dt} = P_K [(H_o) (K_{ib}) + (H_o) (H_{ib}) - (H_o) (H_i) - (H_i) (K_o)]$$

If the external volume is sufficiently large  $H_o$  may be regarded as constant and if the concentration of  $K^+$  is sufficiently small we may put  $K_o = 0$ . We may then write

$$K_{ib} + H_{ib} = C \text{ (which is a constant)}$$

and we have

$$\frac{dH_i}{dt} = P_K H_o (C - H_i)$$

<sup>7</sup> For example, if the internal and external volumes are equal and we put  $H_{ob} = 95$ ,  $H_{ib} = 10$ ,  $K_{ib} = 37.5$  and  $K_{ob} = 0$ , then when 25  $H^+$  has gone in and 25  $K^+$  has come out we have equilibrium and

$$\frac{A_o}{A_i} = \frac{95}{47.5} = 2; \text{ also } \frac{H_o}{H_i} = \frac{70}{35} = 2, \text{ and } \frac{K_o}{K_i} = \frac{25}{12.5} = 2$$

Hence we have at equilibrium

$$\begin{aligned} \frac{dH_i}{dt} &= P_K [(H_o) (K_{ib}) + (H_o) (H_{ib}) - (H_o) (H_i) - (H_i) (K_o)] \\ &= P_K (70) (37.5) + (70) (10) - (70) (35) - (35) (25) \\ &= P_K (0) \\ &= 0 \end{aligned}$$

In dealing with equilibrium  $P_K$  plays no part but it would of course determine the course of the time curve since it is the apparent velocity constant of the process.



Putting  $P_K H_o = V$  we have

$$V = \frac{1}{t} \ln_0 \frac{C}{C - H_i}$$

It is evident that practically all of the K will leave the cell before equilibrium is reached.<sup>8</sup>

If the concentration of K in the external solution is not negligible but the external solution is relatively large the amount of  $K^+$  coming out will make practically no change in the external concentration so that we may regard  $K_o$  and  $H_o$  as constant. We then<sup>9</sup> have (starting with the equation already given)

$$\frac{dH_i}{dt} = P_K [(H_o)(K_{ib}) + (H_o)(H_{ib}) - (H_o)(H_i) - (H_i)(K_o)]$$

<sup>8</sup> If, for example,  $H_{ob} = 100$ ,  $H_{ib} = 0$ ,  $K_{ib} = 10$ , and  $H_o$  is constant we should have at equilibrium  $H_{ib} = 10$ , and in order to arrive at this all of the  $K^+$  inside would have to be replaced by  $H^+$ . Theoretically, however, the entrance of  $H^+$  would diminish  $H_o$  slightly so that  $H_{ib}$  at equilibrium would be slightly less than ten and this slight margin would allow a very small quantity of  $K^+$  to persist inside.

The values at equilibrium would be determined by the values of the anions  $A_o$  and  $A_i$  (see p. 278). In this case  $A_o = 100$  and  $A_i = 10$  so that at equilibrium we should have

$$\frac{A_o}{A_i} = \frac{100}{10} = 10 \text{ and (approximately) } \frac{H_o}{H_i} = \frac{100}{10} = 10$$

The value of  $\frac{K_o}{K_i}$  must also equal 10 but since  $K_o$  and  $K_i$  are exceedingly small they may be neglected and we may put (approximately)

$$\begin{aligned} \frac{dH_i}{dt} &= P_K H_o (K_{ib} + H_{ib} - H_i) \\ &= P_K H_o (10 + 0 - 10) \\ &= P_K H_o (0) \\ &= 0 \end{aligned}$$

<sup>9</sup> To test this numerically let us put  $H_{ob} = 50$ ,  $K_{ob} = 50$ ,  $K_{ib} = 10$ , and  $H_{ib} = 0$ . At equilibrium we have

$$\frac{A_o}{A_i} = \frac{50 + 50}{10} = 10 \text{ and } \frac{H_{ob}}{H_{ib}} = \frac{50}{5} = 10; \text{ also } \frac{K_{ob}}{K_{ib}} = \frac{50}{5} = 10$$

$$= P_K [(H_o) (C) - (H_o) (H_i) - (H_i) (K_o)]$$

$$= P_K H_o \left[ C - H_i - \frac{(H_i) (K_o)}{H_o} \right]$$

$$= P_K H_o \left[ C - \left( 1 + \frac{K_o}{H_o} \right) (H_i) \right].$$

Putting  $1 + \frac{K_o}{H_o} = D$  (a constant) we have

$$\frac{dH_i}{dt} = P_K H_o [C - D(H_i)]$$

and putting  $P_K(H_o) = V$  we have

$$V = \frac{1}{Dt} \ln_e \left[ \frac{C}{C - D(H_i)} \right]$$

In case the external solution is relatively small the case is more complicated but if the permeabilities to the two ions are not very different, so that we may put as an approximation  $P_K = P_H$  (where  $P_H$  is the permeability to H), we can simplify the treatment as the total quantity (not the net amount) of cations passing in any one direction, which we may call  $Q$ , will be approximately constant (since, other things being equal, the exchange of  $K^+$  for  $H^+$  will go on at approximately the same rate as the exchange of  $H^+$  for  $H^+$ , etc., and in any case the total concentration of cations, both inside and outside

We then have

$$\frac{dH_i}{dt} = P_K H_o \left[ K_{i,b} + H_{i,b} - H_i - \frac{(H_i) (K_o)}{H_o} \right]$$

$$= P_K H_o \left[ 10 + 0 - 5 - \frac{(5) (50)}{50} \right]$$

$$= P_K H_o(0)$$

$$= 0$$

remains constant). The total quantity of  $H^+$  passing inward in time will be

$$Q\left(\frac{H_o}{H_o + K_o}\right)$$

and that passing outward will be

$$Q\left(\frac{H_i}{H_i + K_i}\right)$$

and for the net amount passing in we may write

$$\frac{dH_i}{dt} = \frac{Q(H_o)}{H_o + K_o} - \frac{Q(H_i)}{H_i + K_i}$$

Since  $H_o + K_o$  is a constant, we may call it  $A$  and in the same way  $H_i + K_i = B$ , so that we have<sup>10</sup>

$$\begin{aligned}\frac{dH_i}{dt} &= \frac{Q(H_o)}{A} - \frac{Q(H_i)}{B} \\ &= \frac{Q}{AB} [(H_o)B - (H_i)A] \\ &= \frac{Q}{A} \left[ H_o - \frac{A}{B} (H_i) \right]\end{aligned}$$

<sup>10</sup> To illustrate numerically we may put  $H_o = 95$ ,  $K_o = 0$ ,  $K_i = 10$ . When 25  $H^+$  has gone in and 25  $K^+$  has come out we have equilibrium

$$\frac{A_o}{A_i} = \frac{95}{37.5 + 10} = 2, \frac{H_o}{H_i} = \frac{70}{35} = 2, \text{ and } \frac{K_o}{K_i} = \frac{25}{12.5} = 2$$

We then have

$$\frac{H_o + K_o}{H_i + K_i} = \frac{70 + 25}{35 + 12.5} = 2$$

so that

$$\begin{aligned}\frac{dH_i}{dt} &= U_1 [H_o - U_2(H_i)] \\ &= U_1 [70 - 2(35)] \\ &= U_1(0)\end{aligned}$$

Regarding  $Q$  as constant and putting

$$\frac{Q}{A} = U_1 \quad \text{and} \quad \frac{A}{B} = U_2$$

we have

$$\frac{dH_i}{dt} = U_1 [H_o - U_2 (H_i)]$$

If the external solution is well buffered so that  $H_o$  is relatively constant we have

$$U_1 = \frac{1}{U_2} \ln_e \frac{H_o}{H_o - U_2(H_i)}$$

When  $H_o$  is not constant the treatment is still comparatively simple. For convenience the net amount of  $H^+$  which has entered at the time  $t$  is called  $x$  and it is assumed that the internal and external volumes are equal (e.g., 1 liter). Using the subscript  $b$  to denote concentrations at the beginning we have the following:<sup>11</sup>  $H_o = H_{ob} - x$ ;  $K_i = K_{ib} - x$ ;  $K_o = K_{ob} + x$ , and  $H_i = H_{ib} + x$ . Substituting these values in the equation<sup>12</sup>

$$\frac{dx}{dt} = P_K [(H_o) (K_i) - (K_o) (H_i)]$$

we have

$$\begin{aligned} \frac{dx}{dt} &= P_K (H_{ob} - x) (K_{ib} - x) - (K_{ob} + x) (H_{ib} + x) \\ &= (H_{ob}K_{ib} - K_{ob}H_{ib}) - x (K_{ib} + H_{ob} + H_{ib} + K_{ob}) \end{aligned}$$

<sup>11</sup> If the volumes are not equal another coefficient is needed. If, for example, the internal volume is half the external a gram mol of  $H^+$  entering will have twice the concentration inside, so that if we put  $x = 1$  we must put  $H_i = H_{ib} + 2x$ .

<sup>12</sup> Here  $P_K$  is (as before) the permeability to the slower ion  $K^+$ .

Putting  $H_{ob}K_{ib} - K_{ob}H_{ib} = F$  and  $K_{ib} + H_{ob} + H_{ib} + K_{ob} = G$  we have<sup>13</sup> ( $F$  and  $G$  being constants)

$$\frac{dx}{dt} = P_K(F - Gx)$$

$$P_K = \frac{1}{iG} \ln_e \frac{F}{F - Gx}$$

We thus arrive at a more general equation involving no assumptions except those made at the start.

All that is said regarding exchange of cations of course applies equally well to exchange of anions when the cations are indiffusible.

#### SUMMARY

Equations are given for a variety of cases when ions of the same sign are exchanged between the interior of a living cell and the external solution.

<sup>13</sup> To illustrate numerically we may put  $H_{ob} = 95$ ,  $K_{ob} = 0$ ,  $K_{ib} = 37.5$ ,  $H_{ib} = 10$ . At equilibrium  $x = 25$  and we have

$$\begin{aligned} \frac{dx}{dt} &= P_{K_i}(F - Gx) \\ &= P_K [H_{ob}K_{ib} - H_{ib}K_{ob} - x(K_{ib} + H_{ob} + H_{ib} + K_{ob})] \\ &= P_K [(95)(37.5) - (10)(0) - 25(37.5 + 95 + 10 + 0)] \\ &= P_K (0) \\ &= 0 \end{aligned}$$

## THE ACCUMULATION OF ELECTROLYTES

### II. SUGGESTIONS AS TO THE NATURE OF ACCUMULATION IN VALONIA

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It is a familiar fact that electrolytes often appear to contravene physical laws by diffusing against a gradient<sup>1</sup> and attaining a higher concentration inside a living cell than outside.

This happens in the multinucleate cell of the marine alga *Volonia macrophysa*, consisting of a film of protoplasm (only a few microns in thickness) surrounding a large vacuole filled with clear watery sap (outside the protoplasm lies a cellulose wall, but it is so permeable to electrolytes that it need not be considered in this discussion).

Striking differences are seen on opposite sides of the protoplasmic film; for example we find only 0.012 M KCl in the sea water but there may be more than 40 times as much in the sap. We may therefore say that KCl accumulates<sup>2</sup> to a marked degree.

It is natural to enquire whether this can be accounted for by the Donnan principle, but Table I indicates that this is not the case.

<sup>1</sup> In some cases an ion may appear to go against a gradient when in reality this is not the case. It has been shown (Osterhout, W. J. V., and Dorcas, M. J., *J. Gen. Physiol.*, 1925-26, 9, 255) that undissociated CO<sub>2</sub> comes rather quickly into equilibrium across the protoplasm. Let us suppose that owing to differences in pH value the dissociation in the sap is 20 per cent and in the sea water 80 per cent. If the concentration of undissociated CO<sub>2</sub> is the same inside and outside and is called 100, we have for the total inside  $100 + 20 = 120$ , and outside  $100 + 80 = 180$ . If the amount of sea water outside is small and the cell produces enough CO<sub>2</sub> to raise the undissociated CO<sub>2</sub> on each side to 200 we shall have (let us say) 30 HCO<sub>3</sub><sup>-</sup> inside and 160 outside.

<sup>2</sup> The term "accumulation" is used to denote the process by which a penetrating substance reaches a greater concentration inside the cell than outside.

TABLE I\*  
Molar Concentrations

|   | Bermuda sea<br>water | <i>Valonia</i><br><i>macrophysa</i> |
|---|----------------------|-------------------------------------|
|   | M                    | M                                   |
| Cl + Br.....                            | 0.58                 | 0.60                                |
| Na.....                                 | 0.5                  | 0.09                                |
| K.....                                  | 0.012                | 0.5                                 |
| Ca.....                                 | 0.012                | 0.0002?                             |
| Mg.....                                 | 0.057                | Trace?                              |
| SO <sub>4</sub> .....                   | 0.036                | Trace?                              |
| H.....                                  | 10 <sup>-5</sup>     | 10 <sup>-5.8</sup>                  |
| Organic matter, parts per thousand..... |                      | 1.433                               |

\* Cf. Osterhout, W. J. V., *J. Gen. Physiol.*, 1922-23, 5, 225 (where the value of Ca in the sap is erroneously given).

The Donnan principle requires the relation  $K_i \div K_o = Na_i \div Na_o = H_i \div H_o = Cl_i \div Cl_o$  (where the subscripts *i* and *o* refer to inside and outside activities respectively) but these conditions are not fulfilled for according to Table I the observed values<sup>3</sup> are as follows:

$$K_i \div K_o = 0.5 \div 0.012 = 41.60$$

$$Na_i \div Na_o = 0.09 \div 0.5 = 0.18$$

$$H_i \div H_o = 10^{-5.8} \div 10^{-5} = 158.49$$

$$Cl_i \div Cl_o = 0.58 \div 0.6 = 0.97$$

Let us see whether we can employ the Donnan principle to explain the accumulation of K by making certain modifications. We might get rid of the necessity of putting  $K_i \div K_o = Na_i \div Na_o$  by regarding Na as indiffusible since it enters more slowly than K but then we should still be unable to explain the facts for the concentration of indiffusible ions would not be high enough to account for the observed value of  $K_i \div K_o$ . Suppose we regard the Na of the sea water as indiffusible and neglect the counter-effect of the Na inside<sup>4</sup> (i.e., suppose that we

<sup>3</sup> The values can only be approximate since they vary with external conditions so that the ratio of K + Na may be as low as 2.55 or as high as 5.72. For convenience we employ concentrations in place of activities.

<sup>4</sup> Neglecting the counter-effect of the Na inside (about 0.1 M) would more than compensate for neglecting the indiffusible cations outside, i.e., the Mg in excess of SO<sub>4</sub> (about 0.057 - 0.036 = 0.021 M) and the Ca (about 0.012 M) and would therefore favor the attempt to use the Donnan principle more than would be strictly warranted by the facts.

have only KCl inside). We should then have  $(K_0)(Cl_0) = (0.012)(0.58) = 0.007$  and  $(K_i)(Cl_i) = (K_0)^2 = 0.007$  whence  $K_i = 0.084$ . This is far below the observed value of  $K_i$  (which is 0.5) and unless we should assume an impossible concentration of an indiffusible anion inside<sup>5</sup> we could not account for the observed value of  $K_i$  on the Donnan principle.<sup>6</sup>

The difficulty becomes still greater when we consider Cl for if KCl is diffusible we must put at equilibrium  $K_i + K_0 = Cl_0 \div Cl_i$ . Evidently in order that *Valonia* may exist at all the osmotic pressure outside cannot be greater than inside so that  $Cl_0$  cannot be greater than  $Cl_i$ . If we say that  $Cl_i$  must be approximately equal to  $Cl_0$  then at equilibrium  $K_i$  must be approximately equal to  $Cl_0$ .

We escape these difficulties if we abandon the notion of equilibrium and say<sup>6</sup> that KOH and NaOH are constantly entering<sup>7</sup> the cell because their thermodynamic potential is greater outside than inside.

<sup>5</sup> The internal concentration of anions other than Cl is extremely small.

<sup>6</sup> Osterhout, W. J. V., *Proc. Soc. Exp. Biol. and Med.*, 1926, 24, 234; *Bulletin National Research Council*, No. 69, 1929, 170.

<sup>7</sup> The assumption is made later on (p.294) that the entering KOH combines with a weak acid HA produced by the cell. This process may be regarded as practically equivalent to exchanging  $K^+$  for  $H^+$  if we consider only the thermodynamic situation which takes no account of how substances go through the protoplasm. For example (neglecting effects of hydrolysis) we may say that starting with 10 mols of HA inside and 2 of KOH outside and allowing 1 mol of KOH to enter there would be outside 1 of KOH and inside 1 of KA plus 9 of HA; this is equivalent to letting 1 equivalent of  $H^+$  pass out in exchange for 1 of  $K^+$  going in.

In the brief preliminary paper<sup>6</sup> this was not discussed because ionic exchange is regarded as relatively unimportant but it now requires mention since Brooks (Brooks, S. C., *Proc. Soc. Exp. Biol. and Med.*, 1929, 27, 75; *Protoplasma*, 1929, 8, 389; and in *Contributions to Marine Biology*, Stanford University, California, 1930) states that as  $H^+$  tends to pass out and anions cannot accompany it,  $K^+$  comes in to maintain electrical neutrality. But then, in order to explain the behavior of Cl, it is assumed that  $HCO_3^-$  and  $Cl^-$  are diffusible, in direct contradiction to the former assumption. It is said that anions do not travel in the same paths as the cations but this really makes no difference since thermodynamically it does not matter how the ions get through. If  $H^+$  and  $HCO_3^-$  pass out by different paths they can do so simultaneously and thus maintain electrical neutrality without intake of  $K^+$  or  $Cl^-$ . That  $H^+ + HCO_3^-$  can pass rapidly through the protoplasm (as such or in the form of neutral molecules) is shown by the work of M. M. Brooks (*Public Health Reports*, Washington, D. C., 1923, 38, 1470).

The statement that  $HCO_3^-$  exists at a much higher concentration in the sap of *Valonia* than in the sea water is not borne out by the analyses of Dorcas (cf. Osterhout, W. J. V., and Dorcas, M. J., *J. Gen. Physiol.*, 1925-26, 9, 255).



The thermodynamic potential of KOH is higher outside as long as the ionic activity product  $(a_{K_o})(a_{OH_o})$  is greater than  $(a_{K_i})(a_{OH_i})$ . Assuming for convenience that the activity coefficients equal unity we may say that when the pH value of the sap is 5.8 and that of the sea water 8.0, we have  $OH_i = 10^{-8.2}$  and  $OH_o = 10^{-6}$  so that we may write as an approximation  $(K_i)(OH_i) = (10^{-0.3})(10^{-8.2}) = 10^{-8.5}$  and  $(K_o)(OH_o) = (10^{-2})(10^{-6}) = 10^{-8}$ . In order to produce equilibrium we should have to raise the internal concentration of K to 1.585 M (so that  $(K_i)(OH_i) = (10^{0.2})(10^{-8.2}) = 10^{-8}$ ) or raise the internal pH value to 6.3 (so that  $(K_i)(OH_i) = (10^{-0.3})(10^{-7.7}) = 10^{-8}$ ).

Thus we can predict that if we raise the internal pH value higher than 6.3 K should begin to come out, and experiments carried out by Dr. Cooper and Mr. Jacques, in collaboration with the writer, show that this actually happens when the internal pH value is sufficiently raised by the entrance of  $NH_3$ .<sup>8</sup> This, of course, does not apply to NaOH since we have approximately  $(Na_o)(OH_o) = (10^{-0.3})(10^{-6}) = 10^{-6.3}$  and to get equilibrium we should have to raise the internal concentration of Na to the impossible figure of 79.5 M (so that  $(Na_i)(OH_i) = (10^{1.9})(10^{-8.2}) = 10^{-6.3}$ ) or raise the internal pH value to 8.7 (so that  $(Na_i)(OH_i) = (10^{-1})(10^{-5.3}) = 10^{-6.3}$ ). Hence if the internal pH value is raised to the point where K begins to come out Na should continue to go in, as was actually found in the experiments just referred to.

Let us now consider the relative permeabilities to KOH and NaOH. If  $K^+$  and  $OH^-$  penetrate by combining at the surface of the protoplasm to form molecules or enter as the ion pair  $K^+ + OH^-$ , as suggested elsewhere,<sup>9</sup> the quantity passing in will be proportional to the

The hypothesis ignores the fact that the thermodynamic potential of KCl is much higher inside than outside, a matter of vital importance in connection with the inward movement of K which tends to maintain or increase this difference.

There seems to be no ground for assuming that at equilibrium  $H_i$  would be greatly in excess of  $H_o$ , but if it were  $Cl_i$  would have to be greatly in excess of  $Cl_o$ , and  $Cl$  would have to be regarded as indiffusible in order to explain the ratio of  $H_i + H_o$ .

<sup>8</sup> Cf. Cooper, Wm. C., Jr., and Osterhout, W. J. V., *J. Gen. Physiol.*, 1930-31, 14, 117.

<sup>9</sup> Osterhout, W. J. V., *Proc. Soc. Exp. Biol. and Med.*, 1926, 24, 234; *Bulletin National Research Council*, No. 69, 1929, 170; *J. Gen. Physiol.*, 1929-30, 13, 261.

number of collisions and hence to the ionic product<sup>10</sup>  $(K_o)(OH_o)$ : the quantity passing out will be proportional to  $(K_i)(OH_i)$  and the net amount passing, or  $Q_{KOH}$ , will be proportional to the difference of ionic product<sup>11</sup> (which we may call  $p$ ) so that we may put  $p = B [(K_o)(OH_o) - (K_i)(OH_i)]$  where  $B$  is a proportionality factor (which may be omitted in comparing strong electrolytes in the subsequent discussion).

If we define the permeability to KOH (or  $P_{KOH}$ ) as the amount passing through unit protoplasmic surface into the vacuole in unit time with unit difference of ionic product (*i.e.*, when  $p_{KOH} = (K_o)(OH_o) - (K_i)(OH_i) = 1$ ) the amount passing in will be proportional to  $P$ ,  $p$ , surface, and time, so that we may write<sup>12</sup>

$$Q_{KOH} = P_{KOH} p_{KOH} s t \quad \text{and} \quad P_{KOH} = \frac{Q_{KOH}}{p_{KOH} s t}$$

where  $s$  is surface,  $t$  is time, and  $Q$  is the number of gram mols passing in the time  $t$ . We may put as an approximation<sup>13,14</sup>  $p_{KOH} = (K_o)(OH_o) - (K_i)(OH_i) = 0.012 (10^{-6}) - 0.52 (10^{-8.2}) = 87.2 (10^{-10})$ .

In the same way we have for NaOH the value  $p_{NaOH} = (Na_o)(OH_o) - (Na_i)(OH_i) = 0.5 (10^{-6}) - 0.09 (10^{-8.2}) = 4994.3 (10^{-10})$ .

<sup>10</sup> It might seem that the amount entering would be proportional to the difference between the thermodynamic potential inside and outside or to

$$\frac{RT}{F} \ln [(a_{K_o})(a_{OH_o})] - \frac{RT}{F} \ln [(a_{K_i})(a_{OH_i})]$$

but this is not the case (*cf.* van Laar, J. J., *Lehrbuch der theoretischen Elektrochemie*, Wilhelm Engelmann, Leipsic, 1907, pp. 85 ff.).

<sup>11</sup> That  $p$  is analogous to an osmotic pressure gradient is evident from the following: If we assume that  $K^+$  and  $OH^-$  colliding at the outer surface of the protoplasm form molecules of KOH the number produced in unit time will be proportional to  $(K_o)(OH_o)$  and at the inner surface the number will be proportional to  $(K_i)(OH_i)$ . This also applies if we have to do with the formation of an ion pair  $K^+ + OH^-$  (*i.e.*,  $K^+$  and  $OH^-$  entering together).

<sup>12</sup> This resembles the formula of Northrop (Northrop, J. H., *J. Gen. Physiol.*, 1928-29, 12, 435). See also Osterhout, W. J. V., *J. Gen. Physiol.*, 1929-30, 13, 261.

<sup>13</sup> The value is an approximation because we should use activities and also for other reasons. *Cf.* Osterhout, W. J. V., *J. Gen. Physiol.*, 1929-30, 13, 261.

<sup>14</sup> The pH value of the sap is taken as 5.8.

Let us now consider the value of  $Q$ . If a cell is in vigorous growth, increasing 1 per cent a day,<sup>15</sup> the volume of the sap being 1 cc. and containing  $0.52 \text{ M KCl}$  and  $0.09 \text{ M NaCl}$ , the amount of  $\text{K}$  passing in per day may be regarded as  $Q_{\text{KOH}} = 0.52 (10^{-3}) \text{ mol}$  and in the same way  $Q_{\text{NaOH}} = 0.09 (10^{-3}) \text{ mol}$ .

We now have (if  $s$  and  $t$  are the same in both cases)

$$\frac{P_{\text{KOH}}}{P_{\text{NaOH}}} = \frac{Q_{\text{KOH}} \div f_{\text{KOH}}}{Q_{\text{NaOH}} \div f_{\text{NaOH}}} = \frac{0.52 (10^{-3}) \div 87.2 (10^{-11})}{0.09 (10^{-3}) \div 4994.5 (10^{-11})} = 331$$

In other words, the protoplasm appears to be about 331 times as permeable to  $\text{KOH}$  as to  $\text{NaOH}$ .<sup>16</sup>

Not much quantitative significance is to be attributed to these figures and in any case they can apply only where such factors as the movement of  $\text{KCl}$ ,  $\text{NaCl}$ , and ionic exchange can be neglected.

This difference between  $\text{KOH}$  and  $\text{NaOH}$  is greater than would be expected if these substances entered in ionic form with the mobilities now assigned to them. Measurements<sup>17</sup> indicate that if we put the mobility of  $\text{Cl}^-$  in the outermost layer of protoplasm equal to 1, that of  $\text{Na}$  (or  $U_{\text{Na}}$ ) is 0.2; in general, all the anions seem to have about the same mobility so that we may assume that of  $\text{OH}$  (or  $U_{\text{OH}}$ ) to be equal to 1. The mobility of  $\text{K}$  (or  $U_{\text{K}}$ ) is not yet accurately determined, but even if we put it as high<sup>18</sup> as 80 the figures do not correspond to those given above for we have (if we may use the same formula as for aqueous media) for  $D_{\text{KOH}}$ , the diffusion coefficient<sup>19</sup> of  $\text{KOH}$  in the outer layer of the protoplasm,

<sup>15</sup> This rate of growth is realized under favorable conditions.

<sup>16</sup> According to André and Demoussy (André, G., and Demoussy, E., *Bull. Soc. chim. Biol.*, 1925, 7, 899)  $\text{K}$  may diffuse more rapidly than  $\text{Na}$  in the aqueous media of the plant. Even if the permeability to  $\text{K}$  and  $\text{Na}$  were equal and the sap came into equilibrium with the solution bathing the cell, the greater diffusibility of  $\text{K}$  would make the fluid bathing the cells of certain parts of multicellular plants richer in  $\text{K}$  and would thus help to account for the preponderance of  $\text{K}$  in the sap of such cells.

<sup>17</sup> Darnen, E. B., and Osterhout, W. J. V., *J. Gen. Physiol.*, 1929-30, 13, 445.

<sup>18</sup> According to Darnen unpublished results it is less than 80.

<sup>19</sup> Cf. Taylor, H. S., *Treatise on physical chemistry*, D. Van Nostrand Co., New York, 1924, p. 937.

$$D_{\text{KOH}} = RT \frac{2U_{\text{K}} V_{\text{OH}}}{U_{\text{K}} + V_{\text{OH}}} = RT \frac{2(80)(1)}{80+1} = 2RT$$

and for  $D_{\text{NaOH}}$ , the diffusion coefficient of NaOH,

$$D_{\text{NaOH}} = RT \frac{2U_{\text{Na}} V_{\text{OH}}}{U_{\text{Na}} + V_{\text{OH}}} = RT \frac{2(0.2)(1)}{0.2+1} = 0.33 RT$$

Hence

$$\frac{D_{\text{KOH}}}{D_{\text{NaOH}}} = \frac{2}{0.33} = 6$$

According to this the permeability of the outer layer of the protoplasm to KOH is 6 times as great<sup>20</sup> as to NaOH but we have calculated earlier that of the protoplasm as a whole to be about 331 times as great. The calculation which gives the value 6 assumes the penetration of ions only; the other calculation applies whether molecules or ions penetrate but if ions alone penetrate it should agree with the

<sup>20</sup> If it were assumed (*cf.* Brooks, S. C., *Protoplasma*, 1928, 8, 389) that  $\text{H}^+$  inside is exchanged for  $\text{K}^+$  outside and that  $\text{K}^+$  does not tend to pass through the protoplasm more rapidly than  $\text{H}^+$  we might calculate the permeability ( $P_{\text{K}}$ ) to  $\text{K}^+$  as follows: We put

$$P_{\text{K}^+} = \frac{Q_{\text{K}^+}}{p_{\text{K}^+} s t}$$

where  $p_{\text{K}^+} = (\text{K}_o)(\text{H}_i) - (\text{K}_i)(\text{H}_o)$ ,  $Q_{\text{K}^+}$  is the number of equivalents of  $\text{K}^+$  passing in during the time  $t$ ,  $s$  is surface, and  $P_{\text{K}^+}$  (the permeability to  $\text{K}^+$ ) is the number of equivalents of  $\text{K}^+$  entering in unit time when  $s = 1$  and  $p_{\text{K}^+} = 1$ . We should then have approximately (putting  $s = 1$  and  $t = 1$ )

$$\begin{aligned} \frac{P_{\text{K}^+}}{P_{\text{Na}^+}} &= \frac{0.52 (10^{-8}) \div (0.012) (10^{-8}) - (0.52) (10^{-8})}{0.09 (10^{-8}) \div (0.5) (10^{-8}) - (0.09) (10^{-8})} \\ &= 331 \end{aligned}$$

Since unpublished measurements indicate that  $U_{\text{K}}$  in the outer layer is not more than 80 times  $U_{\text{Na}}$  it might be necessary to assume a considerable difference between the concentration of  $\text{K}^+$  and  $\text{Na}^+$  in some part of the protoplasm to give the figure obtained by this calculation.

This calculation agrees with that given on p. 290 since thermodynamically the two cases are alike.

calculation which gives the value 6 and since it does not we may suppose that it is principally molecules that penetrate, or that concentration (and perhaps reaction) in the protoplasm plays a more important rôle than diffusion constants.

It has been shown elsewhere<sup>21</sup> that the mobilities of ions in the protoplasm do not follow the same order as in water. This (and other evidence) points to the fact that the protoplasmic surface is non-aqueous and it therefore seems quite probable that  $K^+$  and  $OH^-$  combine at this surface to pass through as molecules just as  $H^+$  and  $Cl^-$  combine at a water-air surface to pass through the air as molecules and dissociate again on reaching an aqueous medium. For if the surface of the protoplasm forms a non-aqueous layer the concentration of molecules of KOH in this layer will in all probability be much greater than that of the ions  $K^+$  and  $OH^-$  so that, other things being equal, the permeability to molecules will be much greater than the permeability to ions.

For the same reason it does not seem probable that exchange of ions<sup>22,23</sup> plays a great rôle though the cell can of course produce  $H^+$ ,  $NH_4^+$ , and organic anions which are available for exchange.<sup>24</sup>

This point of view is in harmony with the fact that the effective resistance of the protoplasm is high.<sup>25</sup>

<sup>21</sup> Osterhout, W. J. V., *J. Gen. Physiol.*, 1929-30, 13, 715.

<sup>22</sup> In the experiments with  $NH_3$  the amount of K coming out does not correspond to the amount of  $NH_4$  appearing inside and the process does not appear to be ionic exchange (Jacques, A. G., and Osterhout, W. J. V., *J. Gen. Physiol.*, 1930-31, 14, 301).

<sup>23</sup> For the kinetics of ionic exchange see Osterhout, W. J. V., *J. Gen. Physiol.*, 1930-31, 14, 277.

<sup>24</sup> Earlier results with *Nitella* indicating slow ionic penetration were probably due largely to reversible injury (cf. Osterhout, W. J. V., *J. Gen. Physiol.*, 1921-22, 4, 275).

For a discussion of ionic exchange in *Valonia* see Höber, R., and Höber, J., *Arch. ges. Physiol.*, 1928, 219, 269. If the entrance depends on ionic radius (in an aqueous medium) as postulated by Höber, Cs should go in more rapidly than K but this is not the case (cf. Cooper, Wm. C., Jr., Dorcas, M. J., and Osterhout, W. J. V., *J. Gen. Physiol.*, 1928-29, 12, 427).

See also Brooks, S. C., *Protoplasm*, 1929, 8, 389.

<sup>25</sup> Blanks, L. R., *J. Gen. Physiol.*, 1929-30, 13, 361, 495. The effective resistance  $R$  may be regarded as  $R = \frac{E - P}{I}$ , where  $I$  is the current,  $E$  is the e.m.f. producing

The idea that there is little or no penetration of ions is supported by experiments with  $\text{CO}_2$ . It is found<sup>26</sup> that if the concentration of undissociated molecules of  $\text{CO}_2$  or  $\text{H}_2\text{CO}_3$  is kept constant the rate of penetration remains constant even though great variations occur in the concentration of ions. The behavior of the time curves at high and low pH values leads to a similar conclusion.<sup>27</sup>

In this paper we shall for convenience speak of the penetration of electrolytes in molecular form without implying that ionic penetration does not occur but only that it must play a subordinate rôle owing to the non-aqueous character of the protoplasmic surfaces.

These remarks do not affect anything that is said concerning equilibrium since this is in no way dependent on the mode of entrance. But it should be remembered that in a living and growing cell no equilibrium is possible, the nearest approach to it being the sort of pseudo-equilibrium observed with certain rapidly penetrating substances<sup>28</sup> such as  $\text{CO}_2$  and  $\text{H}_2\text{S}$ .

We may sum up by saying that penetration in ionic form would seem to be much slower than in molecular form. If penetration occurs by means of ion pairs (e.g., as  $\text{K}^+ + \text{OH}^-$ ) we should have the equations already given but this would not be the case if it occurs by exchange, e.g., if  $\text{K}^+$  enters in exchange for  $\text{H}^+$  the amount passing through<sup>29</sup> would be proportional to  $(\text{K}_o)(\text{H}_i) - (\text{K}_i)(\text{H}_o)$ .

Let us now consider the distribution of anions. Evidently the

the current (whether an applied potential or one existing in the plant or a combination of both), and  $P$  is the polarization or back E.M.F. produced by the plant during the passage of the current (opposing the passage of the current). In most cases  $P$  accounts for most of the observed resistance.

The effective conductance is  $1 + R$ .

<sup>26</sup> Jacques, A. G., and Osterhout, W. J. V., *J. Gen. Physiol.*, 1929-30, 13, 695.

<sup>27</sup> It is often assumed that if a weak acid penetrates more rapidly at low pH values it must enter chiefly in molecular form but this is not justified since, for example, lowering the pH value increases undissociated  $\text{CO}_2$  or  $\text{H}_2\text{CO}_3$ , and the product  $(\text{H})(\text{HCO}_3)$  in the same proportion (since  $(\text{H})(\text{HCO}_3) = C(\text{H}_2\text{CO}_3)$  where  $C$  is the dissociation constant). See Osterhout, W. J. V., *J. Gen. Physiol.*, 1929-30, 13, 261.

<sup>28</sup> Osterhout, W. J. V., and Dorcas, M. J., *J. Gen. Physiol.*, 1925-26, 9, 255; Osterhout, W. J. V., *J. Gen. Physiol.*, 1925-28, 8, 131.

<sup>29</sup> Cf. Osterhout, W. J. V., *J. Gen. Physiol.*, 1930-31, 14, 277.

penetration of KOH and NaOH would raise the internal pH value if the cell were not continually producing acids (*e.g.*, carbonic and other organic acids).<sup>6</sup> If we lump these acids under the term  $HA^{30}$  we may say that the entering KOH will produce  $K^+ + A^-$  thus increasing the concentration of  $A^-$  if  $HA$  is a weak acid, as is probably the case. As this process continues  $A^-$  will increase and will tend to pass out of the cell<sup>31</sup> either as  $A^-$  or as undissociated  $HA$  which will dissociate on reaching the sea water. If, for example, we imagine a cell having as the only solute in the sap  $KA$  at a concentration of 0.6 M (with a small amount of undissociated  $HA$ ) there will be a tendency for  $A^-$  to go out and for  $Cl^-$  to move in. This could be effected by exchange of  $A^-$  for  $Cl^-$ , passing through the protoplasmic surfaces as such or as undissociated  $HA$  and  $HCl$  (which dissociate again after traversing the surface). In the absence of complications this might go on until  $A_i$  became equal to  $A_o$ .

When Donnan constraints are present the exchange will tend to produce a condition where  $A_i \div Cl_i = A_o \div Cl_o$ . Such constraints are present in *Valonia*<sup>32</sup> for we have in the sea water indiffusible ions such as  $Mg^{++}$  and  $SO_4^{--}$  (the latter in small concentration), which are normally not found in the sap,<sup>33</sup> and  $Ca^{++}$  which, if present at all in the sap, is found only in traces. We also have  $Na^+$  which enters so slowly that it may be regarded as practically indiffusible in comparison with ions which enter rapidly. If  $HCl$  and  $HA^-$  (or  $Cl^-$  and  $A^-$ ) exchange promptly we might expect them to distribute themselves without much delay according to the Donnan principle so that in the absence of complications there would be a tendency to make  $A_i \div Cl_i = A_o \div Cl_o$ .

When the exchange has raised the value of  $(K_i)(Cl_i)$  to the point where it is equal to  $(K_o)(Cl_o)$ , *i.e.*, to  $(0.012)(0.58) = 0.007$ , the difference of thermodynamic potential of  $KCl$  may be regarded as

<sup>30</sup> The concentration of  $HA$  will presumably be small owing to the fact that it is constantly leaving the cell.

<sup>31</sup>  $A^-$  will tend to pass out when  $(H_i)(A_i)$  is greater than  $(H_o)(A_o)$  or  $(K_i)(A_i)$  is greater than  $(K_o)(A_o)$  even though  $A_i$  be less than  $A_o$  (1).

<sup>32</sup> They do not bring the system into Donnan equilibrium because the continual production of acid and other metabolic activities prevent it.

<sup>33</sup> Quoted in W. J. V., *J. Gen. Physiol.*, 1922-23, 5, 225.

approximately zero and any further exchange of HA for HCl (or of  $\text{Cl}^-$  for  $\text{A}^-$ ) will increase the difference of thermodynamic potential of KCl and lessen that of KA (since the value of  $A_o$ , owing to the large external volume, must be very small and in consequence the product  $(K_o)(A_o)$  must be nearly zero). How far the energy of the latter process is available for increasing the difference of thermodynamic potential of KCl is not certain but in any case there must be an abundant supply of energy for this purpose in the metabolism of the cell. This will be discussed in subsequent papers when additional data are presented.

Since K is found in the sap in combination with Cl it is evident that if it really enters as KOH and reacts to form KA there must be an exchange of  $\text{A}^-$  for  $\text{Cl}^-$  or of HA for HCl.

We may sum up by saying that under normal conditions the difference of thermodynamic potential of KOH tends to drive K in<sup>34</sup> and that of KCl tends to drive K out: if the latter be greater<sup>35</sup> metabolic energy would be required to drive K in and this case may have some relation to that described by Straub.<sup>36</sup>

Turning to a consideration of Cl we may say that the difference of thermodynamic potential of KCl tends to drive Cl out and that of NaCl tends to drive Cl in and that these forces seem to be not far from equal.<sup>37</sup>

It may be noted that the production of HA and the exchange of a weak acid for HCl tend to depress the pH value of the sap; on the

<sup>34</sup> The entrance of KOH would temporarily disturb the existing approximate osmotic equilibrium. This might be compared to the well known experiment in which a closed porous pot containing air and fitted with a manometer is placed in an atmosphere of hydrogen. The entrance of hydrogen is more rapid than the exit of air and in consequence the pressure rises temporarily in the pot. The hursting of cells in dilute alcohol may be due to a similar cause.

<sup>35</sup> The difference of thermodynamic potential of KCl is approximately proportional to  $(K_o)(\text{Cl}_o) - (K_e)(\text{Cl}_e) = (0.5)(0.6) - (0.01)(0.58) = 0.2942$ , and that of KOH to  $(K_o)(\text{OH}_o) - (K_e)(\text{OH}_e) = (10^{-2})(10^{-6}) - (10^{-0.3})(10^{-8.2}) = 0.684(10^{-9})$ .

<sup>36</sup> Straub, J., *Rec. trav. chim. Pays-Bas*, 1929, 48, 49.

<sup>37</sup> The difference of thermodynamic potential of KCl is approximately proportional to  $(K_o)(\text{Cl}_o) - (K_e)(\text{Cl}_e) = (0.5)(0.6) - (0.01)(0.58) = 0.2942$ , and that of NaCl to  $(\text{Na}_o)(\text{Cl}_o) - (\text{Na}_e)(\text{Cl}_e) = (0.5)(0.58) - (0.1)(0.6) = 0.23$ .



other hand the entrance of KOH and NaOH tends to raise it. A balance appears to be struck between these antagonistic processes so that the pH value of the sap does not vary widely and this (as suggested elsewhere<sup>33</sup>) may be due to a regulatory process.

We might therefore, as an alternative hypothesis, regard  $H_i$  as approximately constant and since  $H_o$  is constant we could say that the ratio  $H_i \div H_o$  is fixed and therefore produces a constant potential difference<sup>38</sup> across the protoplasm so that all the other ions (whether passing through in ionic or molecular form) will strive to conform to it<sup>39</sup> so that  $K_i \div K_o$  and  $Na_i \div Na_o$  will tend to equal  $H_i \div H_o$ .<sup>40</sup> But this would involve the necessity of making  $Cl_o \div Cl_i$  tend toward the same value which is evidently not possible if the cell is to exist with practically no anion except  $Cl^-$  inside.

It should be emphasized that the normal ratio of  $H_i \div H_o$  (which is far from equilibrium) is maintained at a high value (about 160) by the energy of metabolism which continually produces H<sub>2</sub>A and which maintains the ratio of  $K_i \div K_o$  and  $Cl_i \div Cl_o$ . It is quite possible

<sup>38</sup> This would be small compared with that due to K since the concentration of H is so small, for a change in the pH value of the sea water produces little change in the P.D. across the protoplasm.

<sup>39</sup> The fixed ratio  $H_i \div H_o$  implies a P.D. across the protoplasm which would act on all ions present.

<sup>40</sup> This is in harmony with what has preceded. If, for example, we say that KOH tends to enter until  $(K_o)(OH_o) = (K_i)(OH_i)$  it is equivalent to saying that K tends to enter until  $K_i \div H_i = K_o \div H_o$  since if we put

$$OH_o = \frac{K_o}{H_o} \quad \text{and} \quad OH_i = \frac{K_i}{H_i}$$

we have

$$(K_o) \left( \frac{K_o}{H_o} \right) = (K_i) \left( \frac{K_i}{H_i} \right)$$

whence

$$\frac{K_o}{H_o} = \frac{K_i}{H_i}$$

Hence if the external solution remains constant and the internal pH value is raised so that  $H_i$  is decreased we shall expect K to come out.

that when  $HA$  is once produced the transfer of molecules and ions, *e.g.*, exchange of  $HA$  for  $HCl$  or of  $A^-$  for  $Cl^-$ , follows without further expenditure of energy.

As another alternative it may be suggested that as  $HA$  and  $KOH$  are present in such small amounts they may be neglected and  $KCl$  may be considered to move in as such. But this scheme would not present so clearly the rôle of the pH value of the sap which, as will be shown in subsequent papers, is very important.

Let us now consider how we may picture the process of penetration and growth. The production of a weak acid  $HA$  inside the cell adds to the existing osmotic pressure which is further increased by the entrance of  $KOH$  and  $NaOH$ , transforming  $HA$  to its salts, and under normal conditions water will enter until the internal pressure nearly equals that outside, as the cell wall is too elastic to offer much resistance.

The entrance of water will dilute any electrolytes which have already reached a pseudo-equilibrium with the outside solution and will therefore favor an inward<sup>41</sup> movement.

With the growth of the cell proceeding in this manner the total concentration of electrolytes inside should be approximately constant as appears to be actually the case.

It seems probable that the rate of penetration will be influenced by the activity of the cell (*e.g.*, by production of acid, or of substances which increase osmotic pressure and absorption of water (such as sugars) or of those which promote the expansion of the cell wall (such as carbohydrates); possibly also substances may be formed which directly influence permeability.

Should the activity of the cell (production of acid, etc.) decline the tendency will be to approach a true Donnan equilibrium, with the same ratio of  $K$  to  $Na$  inside and outside.<sup>42</sup> In consequence, the ratio  $K_i \div Na_i$  will fall off and this is actually observed when activity of the cell is lowered by cooling.

The explanation given here is merely a working hypothesis which must be tested experimentally. Many tests have been made during the last five years which support the hypothesis and which will be

<sup>41</sup> The dilution of  $Cl$  inside may thus favor its entrance in exchange for  $A^-$ .

<sup>42</sup> Death leads to a different condition with the inside and outside exactly the same, and injury tends to produce this condition.

discussed in subsequent papers. For the present we may confine our attention to a consideration of experiments with  $\text{NH}_3$  such as have recently been described.<sup>43</sup>

When  $\text{NH}_4\text{Cl}$  is added to the sea water it soon makes its appearance inside where its concentration may become many times as great as outside. According to our hypothesis we should expect undissociated molecules of  $\text{NH}_3$  or  $\text{NH}_4\text{OH}$  to enter more rapidly than ions and this is evidently the case since the pH value of the sap rises rapidly, apparently because the penetration of  $\text{NH}_3$  or  $\text{NH}_4\text{OH}$  is more rapid than the production of acid in the cell: this accords with the fact (shown by analysis) that the total  $\text{NH}_3$  (by which is meant  $\text{NH}_3 +$  undissociated  $\text{NH}_4\text{OH} + \text{NH}_4$ ) enters the cell much more rapidly than is normally the case with K or Na.

According to our hypothesis the entering  $\text{NH}_3$  or  $\text{NH}_4\text{OH}$  combines with  $\text{H}^+\text{A}$  to form  $\text{NH}_4\text{A}$  and at first there is little exchange but after a few minutes  $\text{H}^+\text{A}$  begins to be exchanged for  $\text{Cl}^-$  so that there should be a steady increase of  $\text{NH}_4\text{Cl}$  in the sap. This is found to be the case; as a result of this exchange and of the production of acid by the cell the pH value of the sap soon stops rising since the result of the exchange is to replace the weak acid  $\text{H}^+\text{A}$  by the strong acid  $\text{HCl}$ ; if this went far enough it would cause the internal pH value to fall off again after its original rise (when the cell is first put into sea water containing  $\text{NH}_4\text{Cl}$ ) and such a falling off is commonly observed.<sup>44</sup>

The rise in pH value will affect the entrance of  $\text{KOH}$  and  $\text{NaOH}$ . The penetration of  $\text{KOH}$  should begin to fall off much sooner than that of  $\text{NaOH}$  and as the pH value of the sap continues to rise K should begin to come out while Na continues to enter. This is because the ionic activity products differ as explained on p. 288. The experiments show that this prediction is correct.<sup>45</sup>

<sup>43</sup> Cooper, Wm. C., Jr., and Osterhout, W. J. V., *J. Gen. Physiol.*, 1930-31, 14, 117; Jacques, A. G., and Osterhout, W. J. V., *J. Gen. Physiol.*, 1930-31, 14, 391; in these experiments the cells lived indefinitely and grew better than when no  $\text{NH}_3$  was added.

<sup>44</sup> The rate at which this occurs depends in fact on the rate of entrance of  $\text{NH}_3$  or  $\text{NH}_4\text{OH}$  and that of the exit of  $\text{KOH}$ . It should be remembered that when the pH value of the sap is raised  $\text{CO}_2$  may move inward from the sea water since the  $\text{CO}_2$  equilibrium will be disturbed.

If we now consider effects upon growth we see that even if the rise in pH value due to  $\text{NH}_3$  does not stimulate the production of acid by the cell the rapid entrance of  $\text{NH}_3$  will cause a quicker rise in osmotic pressure than is normally the case. The result will be an increased entrance of water and distension of the cellulose wall. Before  $\text{NH}_3$  is added the entrance of  $\text{KOH}$  and  $\text{NaOH}$  and consequent absorption of water seems to be slow enough to permit the cell wall to stretch and keep pace with the entrance of electrolytes and in consequence the internal concentration of  $\text{Cl}$  remains relatively steady. But  $\text{NH}_3$  (or  $\text{NH}_4\text{OH}$ ) enters much more rapidly than  $\text{K}$  or  $\text{Na}$  so that it seems possible that the cell wall cannot stretch rapidly enough to keep up and in consequence the total concentration of electrolyte should increase somewhat. This is apparently the case. At first the total concentration increases from about  $0.6 \text{ M}$  to about  $0.61 \text{ M}$  after which a balance appears to be struck between the pressure and the elasticity of the cell wall so that the total concentration of electrolyte becomes relatively constant.

This increase of pressure explains in part at least the more rapid growth which we observe when  $\text{NH}_4\text{Cl}$  is present in the sea water. It is also quite possible that  $\text{NH}_3$  contributes to this by increasing permeability.

It is evident from all that has been said that we can predict the behavior of  $\text{K}$  better by disregarding the movement of  $\text{KCl}$  and paying attention to that of  $\text{KOH}$ . It may be remarked incidentally that such evidence as we have<sup>44</sup> (which is not conclusive) indicates that the permeability to  $\text{KCl}$  is not greater than to  $\text{NaCl}$  and that in consequence the movement of  $\text{KCl}$  may be neglected.

The experiments with  $\text{NH}_4\text{Cl}$  seem therefore to support the hypothesis devised to explain what occurs under normal conditions. This hypothesis may be summarized by saying that the energy necessary to produce the accumulation of  $\text{K}$  is furnished by metabolism in connection with the production of a weak organic acid  $\text{HA}$  produced in the cell, and  $\text{HA}$  is exchanged for  $\text{HCl}$  of the external solution (or  $\text{A}$  for  $\text{Cl}$ ) so that it is very much as if the cell produced  $\text{HCl}$  continually and maintained an internal pH value of 5.8 (that of the sea water being about 8). Under these circumstances  $\text{KOH}$  diffuses in to neutralize the acid inside. This tends toward a condition in which

the product  $(K)(OH)$  is the same inside and outside so that if the internal pH value remains 5.8  $K_i$  must become greater than  $K_o$ . If KCl is formed in the sap faster than it comes out there must be an accumulation and this is understandable if KCl passes chiefly in molecular form and permeability to molecules of KCl is low.

This is not an equilibrium condition for if the expenditure of energy stops and acid is no longer produced in the cell the internal pH value will fall and K will come out so that eventually the distribution of electrolytes inside and outside will tend to become nearly equal.<sup>45</sup>

It may be added that experiments on artificial models show that when a difference of pH value is maintained on opposite sides of a partition an electrolyte may pass over from the alkaline to the acid side and accumulate to a marked extent even though no indiffusible ions are present. These experiments will be described in subsequent papers.

#### SUMMARY

It is suggested that K enters chiefly as KOH, whose thermodynamic potential (proportional to the ionic activity product  $(a_K)(a_{OH})$ ) is greater outside than within. As this difference is maintained by the production of acid in the cell K continues to enter, and reaches a greater concentration inside than outside. KOH combines with a weak organic acid which is exchanged for HCl entering from the sea water (or its anion is exchanged for  $Cl^-$ ), so that KCl accumulates in the sap.

Na enters more slowly and its internal concentration remains below that of K.

The facts indicate that penetration is chiefly in molecular form.

As the system is not in equilibrium the suggestion is not susceptible of thermodynamic proof but it is useful in predicting the behavior of K, Na, and  $NH_4$ .

<sup>45</sup> The excess of  $Mg^{++}$  over  $SO_4^{--}$  in the sea water would tend to produce a Donnan equilibrium.

## THE ACCUMULATION OF ELECTROLYTES

### III. BEHAVIOR OF SODIUM, POTASSIUM, AND AMMONIUM IN VALONIA

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As stated<sup>1</sup> elsewhere, we should theoretically expect that when  $\text{NH}_3$  enters the cell and raises the pH value of the sap beyond a certain point K may come out while Na continues to go in.

Experiments carried out by Dr. Cooper and the senior author<sup>2</sup> showed that the entrance of  $\text{NH}_3$  was accompanied by the exit of K but the behavior of Na was left uncertain. It was not realized at that time that the  $\text{K} \div \text{Na}$  ratio in the sap could be so easily affected by external conditions, e.g., by handling the cells or by changing the sea water or by illumination. But in the present experiments conclusive results have been secured by controlling the conditions more carefully, e.g., by keeping the cells continuously in running sea water,<sup>3</sup> avoiding the handling during the experiment and taking care that in each experiment all the cells were equally illuminated.

The effect of light is important since a moderate amount of illumination (but not direct sunlight) promotes growth.<sup>2</sup> For example, when cells were kept side by side in ordinary sea water in the open but not in direct sunlight, one lot in a glass container gained 13 per cent in 20 days but those in a similar container which was painted black outside made no gain.<sup>4</sup> Under natural conditions the cells grow in clumps several layers thick and the illumination therefore varies from

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<sup>1</sup> Osterhout, W. J. V., *J. Gen. Physiol.*, 1930-31, 14, 285.

<sup>2</sup> Cooper, Wm. C., Jr., and Osterhout, W. J. V., *J. Gen. Physiol.*, 1930-31, 14, 117. For effects of light on absorption in *Nitella* see Hoagland, D. R., and Davis, A. R., *J. Gen. Physiol.*, 1923-24, 6, 47.

<sup>3</sup> Brooks, S. C., (*Protoplasma*, 1929, 8, 389) states that the  $\text{K} \div \text{Na}$  ratio is increased by increase or decrease of the normal amount of K in the sea water.

<sup>4</sup> The difference in temperature in the two tubes was probably small.

layer to layer, so that we should anticipate marked variations in a group of cells collected at one time and place.

To this variation in the natural state we must add the possibility of changes brought about by handling. Blinks<sup>5</sup> has shown that in *Valonia ventricosa* and *Nitella* the direct current resistance is reduced to a very low value by mechanical shock, and that even handling is sufficient to produce marked effects which may last a surprisingly long time. All this suggests the necessity for reducing the handling to the minimum, and allowing a period of rest after collection in order to get rid of the effects of pulling the clumps apart.

The first step in our work was the separation of the cells (immediately after collection); this produces a slight softening of most of the cells, and subsequently about 10 per cent die. Fortunately it is possible in Bermuda to get cells free from sponges and algae, so that the problem of cleaning is not troublesome.

TABLE I

|                           | K                | Average | Difference      | Na               | Average | Difference      |
|---------------------------|------------------|---------|-----------------|------------------|---------|-----------------|
|                           | <i>m</i>         |         | <i>per cent</i> | <i>m</i>         |         | <i>per cent</i> |
| <i>A</i>                  | 0.4902<br>0.4897 | 0.4900  | 0.1             | 0.1289<br>0.1296 | 0.1293  | 0.6             |
| <i>B</i>                  | 0.4986<br>0.5004 | 0.4995  | 0.4             | 0.1210<br>0.1200 | 0.1205  | 0.8             |
| <i>C</i> { Large<br>Small | 0.4743<br>0.5111 | 0.4927  | 7.8             | 0.1378<br>0.1138 | 0.1258  | 22.0            |

After separation the cells were stored out of doors (not in direct sunlight) in 3-gallon glass bottles. At intervals the sea water was changed, the cells being disturbed as little as possible during this operation. The sea water was changed daily during the first few days after collection, in order to avoid undue contamination by potassium-rich sap from injured cells, and dead cells were removed as discovered. Subsequently the change was made every 3 to 5 days. The cells were stored under these conditions for at least a month (in some experiments much longer).

The object of this seasoning process was to produce a uniform group of cells. This point was tested in the following way. Cells which had been collected and treated as described above, were divided into 3 groups of the approximate ranges, 0.75-0.9 cc., 0.6-0.4 cc., and 0.4-0.2 cc. Three selections, *A*, *B*, and *C*, were then made, each containing 5 of the large, 15 of the medium, and 20 of the small cells. Selections *A* and *B* were then extracted, each yielded about 12 cc. of sap,

<sup>5</sup> Blinks, L. R., *J. Gen. Phys.* 1, 1929-30, 13, 361, 495.

enough for analysis in duplicate for K and Na in each sample. Selection C was divided into two parts, the one containing enough large and medium cells for a single determination of K and Na, and the other comprising the remainder (small cells). The results are given in Table I (which shows that with lots A and B the difference between duplicate analyses was less than 1 per cent but that the difference between large and small cells is much greater, indicating that the  $K \div Na$  ratio is higher in small cells, *i.e.*,  $K \div Na$  is 3.44 for large and 4.49 for small cells, as would theoretically be expected<sup>1</sup> if the small cells, being younger, are in a more active state). It therefore seemed best to use for each experiment a typical lot of cells, *i.e.*, a lot graded as to size in definite manner, arbitrarily called typical so that any typical lot will be comparable with any other.

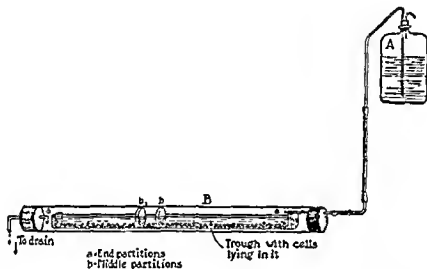


FIG. 1. Tube containing cells exposed to a solution constantly flowing through the tube from the reservoir A. The cells lie in a glass trough with perforated end walls (a) and perforated partitions (b).

In each experiment two typical lots of cells, alike in number and size, were exposed under exactly similar conditions to (a) sea water, and (b) sea water containing a small concentration of  $NH_4Cl$ . A certain number of cells in each group were selected for determination of volume, since it is not desirable to subject all the cells to the handling necessary for such measurements. At intervals cells were withdrawn from each group and the following determinations were made: K, Na,  $NH_3 + NH_4$ , total halide, and pH value. At the same time the changes in volume were measured. The total length of exposure was usually 30 days. The concentration of the  $NH_4Cl$  in the sea water ranged from 0.0025 to 0.001 M.

The work was carried out in Bermuda during the spring of 1930. During the period the lowest temperature recorded was  $16^\circ C.$  and the highest  $26^\circ C.$ , the average temperature was  $21^\circ C.$  The procedure and results for a typical experiment are described in detail below.

A group of 650 cells were selected and divided into two groups, the first con-



sisting of a typical lot, to be used for volume measurement, and the second containing the remainder (also a typical lot). The cells were placed in a pyrex glass trough, which was essentially a half glass tube of about 1 inch radius. This trough was blocked at both ends with paraffin wax partitions, perforated to allow the sea water, but not the cells, to pass through, with two similar partitions ( $b$  and  $b_1$ , Fig. 1) cutting off a portion of the trough near the center. In this portion the volume control cells were exposed, while the other cells occupied the portions on either side. This arrangement was designed to give the volume cells an exposure similar to the average exposure of the other cells. The trough, 34 inches long, was placed in a pyrex tube of  $2\frac{1}{2}$  inches diameter, 36 inches long. The tube was fitted with one-hole rubber stoppers, through one of which was thrust a tube drawn down to a capillary at the end and connected by rubber and glass

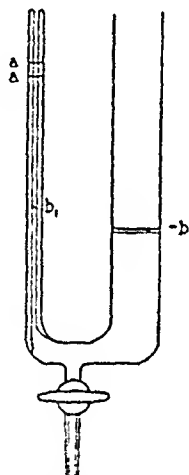


FIG. 2. Apparatus for determining the volume of living cells (explanation in text).

K, Na,  $\text{NH}_3 + \text{NH}_4$ , total balide and pH value. This set of determinations, called hereafter the initial values, serves as the reference point to which all subsequent determinations are referred.

The initial volumes of the two typical lots of cells were next determined in the apparatus shown in Fig. 2. The volume tube was a U-tube with one wide arm and a capillary arm of about 1.5 mm. radius. A stopcock at the bottom was added for convenience in calibrating and cleaning the apparatus. The apparatus was held by a ring stand clamped to a solid table—and readings were made by means of a 1-meter brass scale cathetometer, reading to 0.005 cm. (made by Gaertner). This also was fixed in position.

In making a determination, the volume apparatus was first partially filled with sea water and inserted in its clamps. All the volume cells for the particular determination on hand were then poured out on nicbrome gauze, allowed to drain for a moment, and then transferred one at a time to several layers of filter paper where they were allowed to drain for from 30 to 60 seconds. During this time the level of the meniscus in the capillary arm of the U-tube was read, and the distance between two marks "a" as a check of the mechanical operation of the cathetometer. Finally the cross hairs were set on one of the "a" reference marks. The cells were then dropped in the wide arm of the U-tube, one at a time. When all the cells had been added we first ascertained that the level of the apparatus had not changed (by noting that the cross hairs were still on the reference mark), and determined the change in level in the wide tube by observing the distance that the meniscus had risen in the capillary arm. There was of course a capillary effect but as both the wide and capillary arms of the U-tube had been selected for uniformity it is reasonable to assume that this would be the same for all readings. To make it possible to transform the cathetometer readings to cubic centimeters the tube was calibrated by weighing the distilled water corresponding to changes in the meniscus level for several intervals.

In measuring the volume of the cells the errors involved are, the error of setting the cathetometer, error due to difficulty in determining the bottom point of the meniscus, error due to lack of uniformity in the cross sections of the arms of the U-tubes, and the error due to incomplete drainage of the cells. The first of these errors can be practically eliminated by carefully levelling the telescope at each reading. The second was made negligible by reading the meniscus in the narrow arm where its width is so small that the lowest point can be located with ease. The third error was made small by using in the construction of the apparatus tubes which had been gauged for uniformity. The last error is the most important. It is not desirable to wipe the cells dry with cloth or filter paper, since this procedure has been found to result in the death of a fairly large proportion of them. We have, therefore, to depend on drainage on filter paper to dry them. We have estimated the total error to be expected from all the causes above by making three complete measurements of the same group, wetting them thoroughly between measurements. The measured volumes were: 6.015, 6.040, 6.020, giving

an average of 6.025, a maximum deviation from the mean of 0.42 per cent and an average deviation from the mean of 0.17 per cent. These deviations are negligible compared with the deviations of the analyses due to lack of uniformity of the cells.

During the course of a volume measurement, a cell occasionally died. We allowed for this by measuring the volume of the dead cell at the time of death, and the volume of all the cells, and in subsequent measurements we added the former to the found volume of the remaining cells. It is clear that when this procedure becomes necessary, the measured volume plus the correction for any subsequent point in a run will be too small by an amount equal to growth which the dead cell would have experienced from the time of death until the time of the measurement in question, had it not died. No doubt a correction to the correction could have been estimated, based on the growth of the whole group for any period. But the advisability of attempting this in view of the magnitude of other errors in the work seemed questionable. In the detailed experiment, in fact no volume cells were lost so that error from this cause does not enter. It is estimated that it did not in any case exceed 0.5 per cent.

The analyses for K and Na were made in the following manner: The sap was centrifugalized in covered tubes for 20 minutes to remove chloroplasts and as much as possible of the jelly-like clots which are always present in the expressed sap. Two 5-cc. samples were then taken and evaporated to dryness on the water bath, dried in the oven and finally ignited at 300° to 350°C. to remove all traces of carbon, and in the case of the ammonia group the  $\text{NH}_4\text{Cl}$  also. The residue was weighed and the assumption was made that it was a mixture of NaCl and KCl only. Occasionally a sample of sap was found to have a trace of  $\text{SO}_4$  on testing with acidified  $\text{BaCl}_2$ , and at one time our procedure involved the removal of this and of any possible traces of Mg and Ca before evaporation. A comparison of the results obtained after removal with those obtained without removal suggested that this extra labor was not worth while.

The K was determined as  $\text{KClO}_4$  in the usual way, and the Na was calculated by difference from the weights of the total chlorides and the weight of KCl corresponding to the weight of K-perchlorate found.

A determination of the accuracy of our method of analysis made on a known mixture of K-, Na- and  $\text{NH}_4$ -chlorides resembling sap of cells after exposure, gave the following results:

|         | <i>Found</i> | <i>Actual</i> | <i>Per cent deviation</i> |
|---------|--------------|---------------|---------------------------|
| K.....  | 0.5013       | 0.5000        | 0.26                      |
| Na..... | 0.092        | 0.1000        | 1.8                       |

These results were obtained at the outset of the work when the analytical procedures were unfamiliar, and subsequent results are doubtless better. Nevertheless, since we could use conveniently only 5 cc. of sap for each analysis the weight of sodium chloride is small, in general about 0.04 gram, and this coupled

with necessity for making the weighing of the combined salts very rapidly, in order to prevent the absorption of weighable amounts of water in the very moist atmosphere of Bermuda, throws a considerable burden on the accuracy of the Na analysis.

The halide was determined by Mohr titration with  $\text{AgNO}_3$ , on a duplicate pair of 1-cc. samples.

The pH value was determined in a single 1-cc. sample as a rule, but occasionally the sap of a single cell was compared against a like volume of buffer solution. Chlorophenol red was the indicator used.

A single analysis for ammonia was made on the sap of the cells before exposure. It was found that the amount present was less than could be determined satisfactorily by our usual method. A trial later on other unexposed cells, using the much more delicate Nessler's reagent, indicated that there is less than 0.0005 mols per liter normally present in cells which have been stored in changes of sea water obtained far enough from land to be free of contamination.

The usual method of determining ammonia was distillation of the free ammonia into a known amount of standard acid, and determination of the amount used up by titration of the unused acid with standard  $\text{CO}_2$ -free alkali using methyl red as indicator. An estimation of the accuracy of the method in our hands was made by determining ammonia on a known sample containing 0.0500 mol of ammonium chloride per liter. The results are:

|                     | Found  | Actual | Per cent deviation |
|---------------------|--------|--------|--------------------|
| $\text{NH}_4$ ..... | 0.0500 | 0.0510 | 2.0                |

(Duplicate 1-cc. samples were used for ammonia determination.)

Tables II and III and Fig. 3 show the results of an experiment in which sufficient 0.6 M  $\text{NH}_4\text{Cl}$  was added to bring the concentration of  $\text{NH}_4\text{Cl}$  in the sea water up to 0.001 M.

If we suppose, as stated elsewhere,<sup>1</sup> that KOH enters,<sup>6</sup> and assume, for convenience, that the activity coefficients equal unity<sup>7</sup> we may say as an approximation that equilibrium will be reached when  $(K_o)(\text{OH}_o) = (K_i)(\text{OH}_i)$ , where the subscripts *i* and *o* refer to the activities (concentrations) inside and outside. Under normal conditions (when the pH value of the sea water is 8 and that of the sap 5.9) we have  $(K_o)(\text{OH}_o) = (10^{-2})(10^{-6}) = 10^{-8}$ . In order to produce equilibrium

<sup>6</sup> Cf. Osterhout, W. J. V., *J. Gen. Physiol.*, 1929-30, 13, 261.

<sup>7</sup> This makes practically no change in the present case where only relative activities and concentrations are considered, i.e., where different activities or concentrations are compared.

TABLE II  
*Changes in the Sap of Valonia Produced by Exposure to Sea Water at pH 8 Containing 0.001 M  $\text{NH}_4\text{Cl}$*

| Days | Control group           |        |                   |        |     |               | Ammonia group           |        |                                      |                   |               |       |        |
|------|-------------------------|--------|-------------------|--------|-----|---------------|-------------------------|--------|--------------------------------------|-------------------|---------------|-------|--------|
|      | Molecular concentration |        |                   |        | pH  | Volume<br>cc. | Molecular concentration |        |                                      |                   | Volume<br>cc. | pH    |        |
|      | K                       | Na     | Total*<br>cations | Halide |     |               | K                       | Na     | NH <sub>3</sub> +<br>NH <sub>4</sub> | Total*<br>cations |               |       | Halide |
|      |                         |        |                   |        |     |               |                         |        |                                      |                   |               |       |        |
| 0    | 0.4922                  | 0.1338 | 0.6260            | 0.6092 | 5.9 | 5.450         | 0.4922                  | 0.1338 | 0.0005                               | 0.6260            | 0.6092        | 5.125 | 5.9    |
| 2    | 0.4905                  | 0.1276 | 0.6181            | 0.6070 | 5.9 | 5.570         | 0.4714                  | 0.1345 | 0.0166                               | 0.6225            | 0.6170        | 5.265 | 6.4    |
| 5    | 0.4916                  | 0.1317 | 0.6233            | 0.6088 | 5.9 | 5.635         | 0.4557                  | 0.1468 | 0.0297                               | 0.6322            | 0.6183        | 5.400 | 6.0    |
| 7    | 0.4980                  | 0.1245 | 0.6225            | 0.6164 | 5.9 | 5.660         | 0.4328                  | 0.1481 | 0.0390                               | 0.6199            | 0.6190        | 5.555 | 6.2    |
| 10   | 0.4909                  | 0.1266 | 0.6175            | 0.6158 | 5.9 | 5.665         | 0.4160                  | 0.1551 | 0.0592                               | 0.6303            | 0.6164        | 5.675 | 6.0    |
| 15   | 0.4902                  | 0.1269 | 0.6171            | 0.6183 | 5.9 | 5.670         | 0.3963                  | 0.1581 | 0.0801                               | 0.6345            | 0.6208        | 5.800 | 6.2    |
| 20   | 0.4921                  | 0.1252 | 0.6173            | 0.6149 | 5.9 | 5.685         | 0.3675                  | 0.1469 | 0.1119                               | 0.6263            | 0.6202        | 5.900 | 6.2    |
| 30   | 0.4906                  | 0.1299 | 0.6205            | **     | 5.9 | 5.720         | 0.3579                  | 0.1497 | 0.1252                               | 0.6328            | **            | 5.950 | 6.0    |

\* The excess of total cations over halide has been discussed in previous work (Cf. Osterhout, W. J. V., and Dorcas, M. J., *J. Gen. Physiol.*, 1924-25, 7, 633).

\*\* Analyses made on single samples and halide omitted owing to insufficiency of cells.

we should have to raise the pH value of the sap to 6.3 (so that  $(K_i)(OH_i) = (10^{-0.3})(10^{-7.7}) = 10^{-8}$ ). In other words, if we raise the pH value of the sap above 6.3 we should expect K to come out because the thermodynamic potential inside, which is proportional to  $(K_i)(OH_i)$ , would be greater than outside.

Fig. 3 shows that K comes out and Table II shows that the pH value of the sap has risen<sup>2</sup> as the result of penetration of  $NH_3$  to about 6.3, but this value is probably too low as previous determinations<sup>2</sup> gave 6.6. It may be said that the determinations of pH value are

TABLE III  
*Gram Mols in Sap*

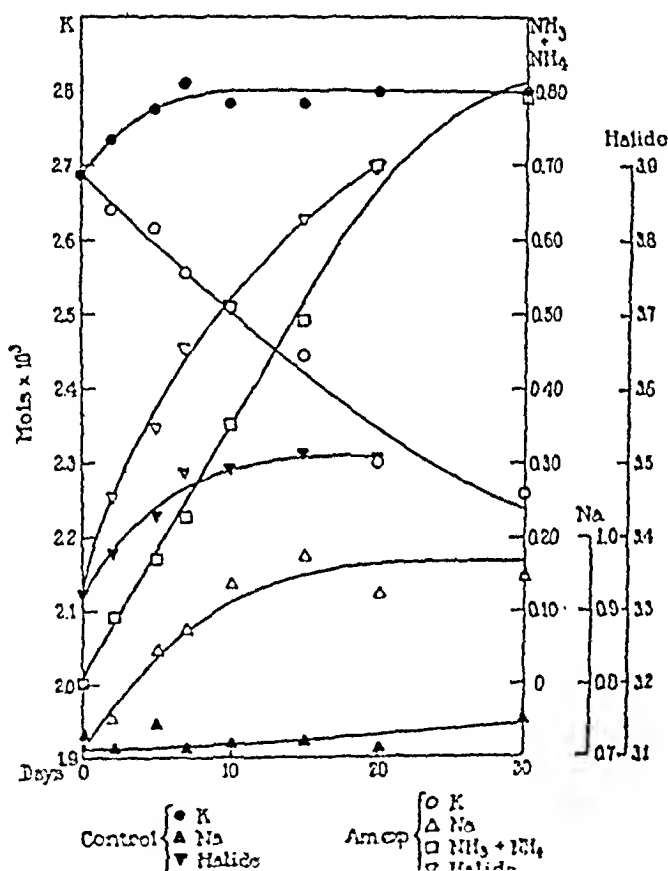
| Days | Gram mols ( $10^3$ ) = concentration $\times$ volume in cc. |        |        |                   |       |        |               |        |
|------|---|--------|--------|-------------------|-------|--------|---------------|--------|
|      | Control   |        |        | Ammonia group     |       |        |               |        |
|      | K   | Na     | Halide | Corrected volumes | K     | Na     | $NH_3 + NH_4$ | Halide |
| 0    | 2.683   | 0.7293 | 3.320  | 5.450             | 2.683 | 0.7293 | 0.00273       | 3.320  |
| 2    | 2.733   | 0.7108 | 3.382  | 5.599             | 2.640 | 0.7530 | 0.09294       | 3.456  |
| 5    | 2.770   | 0.7422 | 3.430  | 5.743             | 2.617 | 0.8430 | 0.17057       | 3.550  |
| 7    | 2.818   | 0.7407 | 3.490  | 5.907             | 2.555 | 0.8750 | 0.23050       | 3.656  |
| 10   | 2.782   | 0.7161 | 3.488  | 6.036             | 2.510 | 0.9360 | 0.35630       | 3.720  |
| 15   | 2.780   | 0.7197 | 3.507  | 6.167             | 2.448 | 0.9750 | 0.49400       | 3.828  |
| 20   | 2.797   | 0.7117 | 3.497  | 6.275             | 2.305 | 0.9218 | 0.70220       | 3.891  |
| 30   | 2.806   | 0.7430 | —      | 6.327             | 2.260 | 0.9472 | 0.79220       | —      |

not very accurate owing to the high concentration of salt and the fact that the sap is practically unhuffed.

Let us now consider the behavior of Na. The external ionic product is  $(Na_e)(OH_e) = (10^{-0.3})(10^{-6}) = 10^{-6.3}$  which is greater than the internal or  $(Na_i)(OH_i) = (10^{-0.87})(10^{-7.7}) = 10^{-8.57}$  even when the pH value of the sap is 6.3 (*i.e.*, when  $OH_i = 10^{-7.7}$ ). Hence we should expect Na to go in. As a matter of fact Na does continue to penetrate and at a greater rate than before.<sup>8</sup> This might be attributed to injury but as a matter of fact these cells lived indefinitely and grew better than cells not exposed to ammonia and microscopic examination

<sup>8</sup> It happens that these controls show very slight gain in mols of Na in 30 days but in other experiments there was more.

showed nothing abnormal. It might, however, be thought that a few injured cells which allowed Na to enter and K to come out were responsible for the result. This seems improbable in view of the



not above the normal. It may be added that similar results have been gotten repeatedly in other experiments which would imply the same degree of injury in all cells exposed to ammonia (if the result were attributed to injury).

The rate of penetration of Na would tend to rise above the normal due to the dilution of electrolytes inside caused by the entrance of water (which is more rapid than normal as shown by the increased rate of growth) but on the other hand the rise in the pH value of the sap would tend to depress the rate since it would decrease the driving force (which is proportional to the difference between the external and internal thermodynamic potential).

The increased rate of entrance of Na may be due to an increase in permeability caused by  $\text{NH}_3$  and this would harmonize with the fact that the exit of K is more rapid than would be expected from its normal rate of penetration. We note that  $(0.002806 - 0.002683 =) 0.000123$  mol of K entered the controls but in the same period the exit of K from the cells exposed to ammonia amounted to  $(0.002683 - 0.002260 =) 0.000423$  or more than 3 times as much. Before concluding that this is due to increase in permeability we must consider the ionic products. If we neglect the effect of the cell wall (as seems justified in the case of *Valonia macrophysa* under normal conditions, on account of the great elasticity of the wall) we may put,<sup>9</sup> as stated elsewhere,

$$P_{\text{KOH}} = \frac{Q_{\text{KOH}}}{p_{\text{KOH}}^{1/2}}$$

where  $P_{\text{KOH}}$  is the permeability<sup>9</sup> to KOH,  $Q$  is the amount passing in during the time  $t$ , and  $s$  is surface. For the entrance of K during 30 days the value of  $Q$  is 0.000123 mol, and for comparative purposes we may put  $s = 1$  and  $t = 1$ . The value<sup>10</sup> of  $p$  is taken as  $(\text{K}_o)(\text{OH}_o) - (\text{K}_i)(\text{OH}_i) = (10^{-2})(10^{-6}) - (10^{-6.3})(10^{-8.1}) = 6(10^{-9})$ . We then have

$$P_{\text{KOH}} = \frac{0.000123}{6(10^{-9})} = 20500$$

<sup>9</sup> I.e., the amount passing through the protoplasm in unit time, through unit surface with unit value of  $p$  (i.e., when  $(\text{K}_o)(\text{OH}_o) - (\text{K}_i)(\text{OH}_i) = 1$ ).

<sup>10</sup> This is an approximation, since strictly speaking we should employ activities, and for other reasons. Round numbers are here employed.



In the same way for the exit of K, when the pH value of the sap is taken as 6.6 and the concentration of K in the sap is given for convenience an average value for the whole period of 0.43  $\pi$ , the value of  $p_{\text{KOH}}$  is  $(K_i)(\text{OH}_i) - (K_o)(\text{OH}_o) = (10^{-0.37})(10^{-7.4}) - (10^{-2})(10^{-6}) = 0.7 (10^{-6})$ . Hence for the exit of K we have (neglecting any difference in  $s$ )

$$P_{\text{KOH}} = \frac{0.000423}{0.7(10^{-6})} = 60429$$

According to this the permeability during the exit<sup>11</sup> exceeds the permeability during the entrance by  $(60429 \div 20500) = 2.9$  times. These figures indicate a decided increase in permeability but their quantitative significance is not very great as some of the data are uncertain<sup>12</sup> and, moreover, a part of the increase might be due to enlargement of the surface.

It might be suggested that the rapid exit of K is due to an exchange of  $\text{K}^+$  for  $\text{NH}_4^+$  but if the entrance of  $\text{NH}_4^+$  depended on such exchange we should not find the rate of entrance increasing with the external pH value<sup>13</sup> as is actually observed.<sup>2</sup> We note also that the amount of ammonia entering is considerably less than the amount of K coming out: for example, in the first two days 0.0208 mol of K has come out and only 0.0161 mol of  $\text{NH}_4$  has gone in.

An exchange of  $\text{K}^+$  for  $\text{Na}^+$  might be assumed but this would not be a rapid process.

Moreover the internal pH value would not rise (as it is seen to do) if only  $\text{NH}_4$  and not  $\text{NH}_3$  or  $\text{NH}_4\text{OH}$  went in; nor would the total halide increase.

It may be of interest to calculate permeability to NaOH going in

as compared with that to KOH coming out. We have (taking the figures for 7 days and using average values for  $K_i$  and  $Na_i$  during this period)

$$\begin{aligned}\frac{P_{KOH}}{P_{NaOH}} &= \frac{Q_{KOH \text{ outward}} \div P_{KOH \text{ outward}}}{Q_{NaOH \text{ inward}} \div P_{NaOH \text{ inward}}} \\ &= \frac{0.000128 \div [(0.463) (10^{-7.4}) - (10^{-2}) (10^{-6})]}{0.0001457 \div [(0.5) (10^{-6}) - (0.141) (10^{-7.4})]} \\ &= 51.7\end{aligned}$$

This is much less than the value of 331 calculated elsewhere for normal conditions<sup>1</sup> when both KOH and NaOH are entering.

Under normal conditions we cannot calculate permeability to KCl since the internal ionic product is greater than the external but in this case if we suppose KCl to pass out we can compare the value of  $P_{KCl}$  going out with  $P_{NaCl}$  coming in. We have

$$\begin{aligned}\frac{P_{KCl}}{P_{NaCl}} &= \frac{Q_{KCl \text{ outward}} \div P_{KCl \text{ outward}}}{Q_{NaCl \text{ inward}} \div P_{NaCl \text{ inward}}} \\ &= \frac{0.000128 \div [(0.463) (0.618) - (0.01) (0.58)]}{0.0001457 \div [(0.5) (0.58) - (0.141) (0.618)]} \\ &= 0.64\end{aligned}$$

This has not much quantitative significance since it involves the assumption that the per cent of Na moving in as NaCl is the same as the per cent of K moving out as KCl.

A similar calculation for the figures at the end of 30 days gives  $P_{KCl} \div P_{NaCl} = 1.5$ . Hence if the calculations mean anything they signify that in the experiments with ammonia permeability to KCl is about the same as to NaCl.

In considering the increase in permeability due to the presence of ammonia we may recall Loeb's<sup>14</sup> suggestion that the protoplasmic surface may resemble a soap whose viscosity depends on the cation (thus  $NH_4$  makes a softer soap than K, also K a softer soap than Na, and Na a softer soap than Ca).

It is also possible that the presence of one substance in the surface layer of the protoplasm may affect the solubility of other substances.

<sup>14</sup> Loeb, J., *The dynamics of living matter*, The Macmillan Co., New York, 1906

It may be desirable to add a word regarding the increased growth in the presence of ammonia.<sup>2</sup> Theoretically this would be expected<sup>1</sup> if we suppose that  $\text{NH}_3$  or  $\text{NH}_4\text{OH}$  on entering combines with a weak organic acid  $\text{HA}$  changing it to  $\text{NH}_4^+ + \text{A}^-$  and so increasing osmotic pressure. This would also happen with the entrance of  $\text{KOH}$  under normal conditions (in absence of ammonia) but much more slowly for it is evident that the entrance of  $\text{KOH}$  is much slower than that of ammonia.

It is also possible that ammonia aids growth in other ways, *e.g.*, by increasing the production of acid in the cell or of substances which promote the expansion of the cell wall or increase permeability.

In view of the more rapid increase of osmotic pressure due to the entrance of ammonia it would not be surprising if the cell wall failed to stretch rapidly enough to keep up so that the total electrolyte inside would increase. This seems to happen<sup>2</sup> as shown in Table II.

The fact that the pH value does not rise beyond a certain point (*cf.* Irwin,<sup>15</sup> Brooks<sup>16</sup>) may be due to the production of acid on the part of the cell and this might possibly take place in a regulatory manner.

As stated elsewhere<sup>1</sup> we assume that there is an exchange of  $\text{HA}$  for  $\text{HCl}$  (or of  $\text{A}^-$  for  $\text{Cl}^-$ ) of the sea water so that there is an accumulation of  $\text{NH}_4\text{Cl}$ : this would aid in keeping down the pH value as it is in effect the exchange of a weak for a strong acid.

#### SUMMARY

When 0.001 M  $\text{NH}_4\text{Cl}$  is added to sea water containing *Valonia macrophysa* there seems to be a rapid penetration of undissociated  $\text{NH}_3$  (or  $\text{NH}_4\text{OH}$ ) which raises the pH value of the sap so that the thermodynamic potential of  $\text{KOH}$  becomes greater inside than outside and in consequence K leaves the cell:  $\text{NaOH}$  continues to go in because its thermodynamic potential is greater outside than inside.

$\text{NH}_4\text{Cl}$  accumulates, reaching a much higher concentration inside than outside. This might be explained on the ground that  $\text{NH}_3$ , after entering, combines with a weak organic acid produced in the cell whose anion is exchanged for the  $\text{Cl}^-$  of the sea water, or (more probably) the organic acid is exchanged for  $\text{HCl}$ .

<sup>15</sup> Irwin, M., *J. Gen. Physiol.*, 1925-26, 9, 235.

<sup>16</sup> Brooks, M. M., *Fish. Health Reports*, Washington, D. C., 1923, 38, 2674.

## ANNOUNCEMENT

### CORRECTION

In foot-note 25, on pages 292-293, Vol. 14, No. 2, November, 1930, the first line should read: "The effective resistance is  $\frac{E}{I}$ ; the ohmic resistance." The last line of this foot-note, reading "The effective conductance is  $1 \div R$ ," should be omitted.

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# THE ORDER OF DEATH OF ORGANISMS LARGER THAN BACTERIA

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(Accepted for publication, November 5, 1930)

In two papers in the preceding volume of this Journal,<sup>1</sup> the author has tried to show that the logarithmic order of death in bacteria can be accounted for by assuming in each cell one or several especially sensitive vital molecules, the inactivation of any one of which will prevent multiplication of the cell.

The order in which organisms would die is different whether 1, 2, 3, etc., molecules must be destroyed before the organism is dead. A computation gave the result that if death is caused by inactivation of one molecule, the order of death is logarithmic, and the logarithms of survivors, plotted against time, show a rectilinear function. If two or more molecules must be inactivated to cause death, the logarithmic survivor curve is "hulging" above the straight line, and there is a short period of no deaths which does not exist if the inactivation of one certain molecule is sufficient to cause death.<sup>2</sup>

The formula for the order of death developed is probably more complicated than the one given in the first paper,<sup>3</sup> but is correct for one reacting molecule.

It has been shown that bacteria die in the logarithmic order which indicates that the inactivation of *one* certain molecule in the cell causes death. The second contribution dealt with exceptional survivor curves of bacteria, and tried to show that they are also essentially logarithmic.

<sup>1</sup> Rahn, Otto, *J. Gen. Physiol.*, 1929-30, 13, 179, 395.

<sup>2</sup> Rahn, Otto, *J. Gen. Physiol.*, 1929-30, 13, 396, Fig. 1.

<sup>3</sup> According to a letter by Dr. A. W. Cressman, randomness should be considered, and it should also be included that the probability of molecules reacting is less for injured cells than for uninjured.

The existence of a logarithmic order has been contested by a number of biologists. To settle this point, Table I brings a compilation of all

TABLE I  
*Survey of Experiments on the Order of Death of Bacteria*

| Author   | Number of experiments      |                 |          |            |
|--|----------------------------|-----------------|----------|------------|
|  | With initial count missing | With death rate |          |            |
|  |                            | Decreasing      | Constant | Increasing |
| Death by Drying                                    |                            |                 |          |            |
| Paul (1909).....                                   | 4                          | —               | —        | —          |
| Paul, Birstein, and Reuss (1910 <i>a</i> ).....    | 10                         | —               | —        | —          |
| Madsen and Nyman (1907).....                       | 2                          | —               | —        | —          |
| Death by Heat                                      |                            |                 |          |            |
| Chick (1910).....                                  | 21                         | —               | —        | —          |
| Reichenbach (1911).....                            | —                          | 3               | 9        | 2          |
| Eijkman (1912-13).....                             | —                          | 3               | 4        | 2          |
| Sattler (1928).....                                | —                          | 62              | 1        | 8          |
| Death by Chemical Poisoning                        |                            |                 |          |            |
| Madsen and Nyman (1907).....                       | 13                         | —               | 8        | —          |
| Chick (1908).....                                  | 12                         | 5               | 2        | —          |
| Chick (1910).....                                  | 6                          | —               | —        | 3          |
| Reichenbach (1911).....                            | —                          | —               | 1        | 2          |
| Paul, Birstein, and Reuss (1910 <i>b, c</i> )..... | 72                         | —               | —        | —          |
| Eijkman (1912-13).....                             | —                          | 1               | —        | 1          |
| Lee and Gilbert (1918).....                        | —                          | 2               | 2        | —          |
| Winslow and Falk (1926).....                       | —                          | 5               | 4        | 2          |
| Myers (1929).....                                  | —                          | —               | 1        | 17         |
| Death by Irradiation                               |                            |                 |          |            |
| Clark and Gage (1905).....                         | —                          | 1               | —        | —          |
| Lee and Gilbert (1918).....                        | —                          | 1               | —        | —          |
| Gates (1929).....                                  | —                          | —               | —        | 2          |
| Total in each group.....                           | 140                        | 83              | 32       | 39         |

the more extensive experiments on the order of death of bacteria known to the author. The death-rate  $K$  is computed from the formula

$$K = \frac{1}{t} [\log (\text{initial number}) - \log (\text{survivors})]$$

The rate is constant for one reacting molecule, and increases if more than one molecule must be inactivated to cause death.

Experiments in which the initial number of cells is unknown, cannot be used as a proof either for or against the logarithmic order, because they give no information about the most important part of the survivor curve, *i.e.*, the possible period of no deaths.<sup>4</sup>

Of the 154 experiments remaining, only thirty-two have a constant death-rate indicating one reacting molecule. The majority, 83, show a decreasing death-rate, or a sagging survivor curve. It has been demonstrated<sup>2</sup> that this indicates one reacting molecule, but a mixture of individuals differing in resistance.

The few cases with increasing death-rates seem to indicate that more than one molecule must be destroyed to cause death. But the alternative explanation that these cases, observed mostly with staphylococci and spore formers, are due to a clustering of the organisms in the plate count method seems more probable.<sup>5</sup>

Some recent experiments with light of short wave-lengths point in the same direction.

One series of experiments, made by Holweck (1929), described by Lacassagne (1929) and interpreted mathematically by Curie (1929) was carried out with a 3 hours old broth culture of *Ps. pyocyanea* spread on an agar surface, and irradiated with x-rays. With light of 4 Å.u. wave-length, the number of survivors decreased in exponential ratio to the dose, which indicates that a single quantum is sufficient to kill the cell. With rays of 8 Å.u. wave-length, 4 quanta are required to kill the cell. No data are given, only two survivor curves, and these not logarithmic. Holweck computes from his experiments that the bacterium has a "sensitive zone" of 0.43 μ diameter in the one and of 0.55 μ diameter in the other experiment. If bacteria are hit by quanta outside this zone, they do not seem to be killed. The average dimensions of the cells are given as 0.7 x 4.0 μ. This sensitive zone corresponds quite well with the author's assumption of one reacting molecule.

Mme. Curie computes a general probability curve for survival of organisms if different numbers of quanta are required for death. If one quantum is sufficient to kill, she finds the proportion of survivors

$$P = e^{-Kt}$$

or

$$-Kt = \log (\text{survivors})$$

which corresponds with the author's formula.

<sup>4</sup> Rahn, Otto, *J. Gen. Physiol.*, 1929-30, 13, 202.

<sup>5</sup> Rahn, Otto, *J. Gen. Physiol.*, 1929-30, 13, 405.



Wyckoff and Rivers (1930) studied the influence of cathode rays upon bacteria and found that a hit by one electron is sufficient to kill a cell of *Bact. coli* or *Bact. aertryke*, but the same did not hold true with staphylococcus because the microscopic investigation showed that this organism was clustered even in very high dilutions. This verifies the author's explanation that increasing death-rates and bulging survivor curves in bacteria are due to clustering, and not to a larger number of reacting molecules per cell. Wyckoff and Rivers proved this by incubating a plate inoculated with *Bact. coli* before exposure to cathode rays; the result was a strongly bulging survivor curve.

There can be no doubt that the number of molecules in a cell is quite large. A cell of *Streptococcus lactis* ferments about  $12 \times 10^{-10}$  mg. of sugar per hour, or one million molecules per second. This fermentation must require a considerable number of zymase molecules. But the essential growth mechanism is condensed in the chromosomes which are but a very small part of the cell, and the chromosomes are believed to be subdivided into genes each of which represents a special property, *i.e.*, they are all different chemically. Probably, the inactivation of only one such gene would prevent growth.

The number of different genes is so large and the space, especially in bacteria cells, so small, that each gene can consist only of a very few molecules.

Allowing, then, that the evidence permits the assumption that death of bacteria is brought about by the destruction of only one definite gene-like molecule in the cell, it is of greatest interest to see what this status would be with other organisms. Only very few facts on death-curves of other organisms are available, and we shall analyze them beginning with the simplest forms.

Of all unicellular organisms, the yeasts are usually considered to be the closest relatives of bacteria. There have been several experiments published on the death of yeast. Eijkman (1912-13) gives no data, but merely the survivor curves which are copied in Fig. 1 drawn to standard scale as well as that is possible. These curves, together with Eijkman's statement that yeasts always give this type of curve, suggest that more than one molecule must be destroyed in yeast before the cell loses the power of multiplication, unless Eijkman's yeast showed clumping.

The paper of Fulmer and Buchanan (1923) on the death of yeast by chemical poisons also brings only curves and no data. The indicator

for death in these experiments was the staining with methylene blue. This means a new definition of death. All previous discussion was based on the standard plate count method of bacteriology, and this implies the definition that a cell is dead if it loses the power of multiplication. Fulmer and Buchanan define a cell as dead if it turns blue in methylene blue solution. We have no definite knowledge of the relation between these two reactions. It seems most probable, at least to the author, that increased permeability indicates a very late stage in the death process of a cell while loss of multiplying power is probably one of the first stages. There are many intermediate stages between the active, living cell and the absolutely dead cell. We

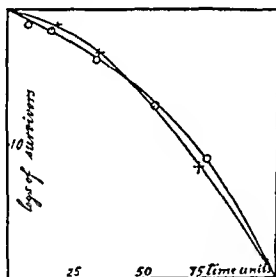


FIG. 1. Order of death of a pink yeast on standard scale.

know, *e.g.*, that yeast cells which have lost permanently the power to multiply may still be able to ferment, and such cells will not take the stain. If the symptoms of death as defined and measured by Fulmer and Buchanan do not appear until one or two minutes after the loss of multiplication, a "period of no deaths" must be expected. The time difference between the two symptoms of death has not been determined and therefore, the survivor curves given by these authors cannot be used for interpretation of the essential point. It may just be summarized that about one-third of these curves suggests a logarithmic order of death, while 18 per cent are bulging, 15 per cent have 2 maxima, and the others are irregular.

Sattler's data (1928) on the death of pink yeast by heat are too incomplete for conclusions to be drawn, the yeast dying too fast at the pasteurizing temperature of milk. It is therefore impossible to state accurately the order of death of yeast until more data are available.

Mr. J. A. Woerz has studied in this laboratory the order of death by heat of a small yeast, *Torula cremoris*, which does not cluster, and he found the logarithmic order well established at temperatures considerably higher than the maximum temperature of growth. At temperatures very near the maximum, a complication sets in which we have not as yet been able to account for.

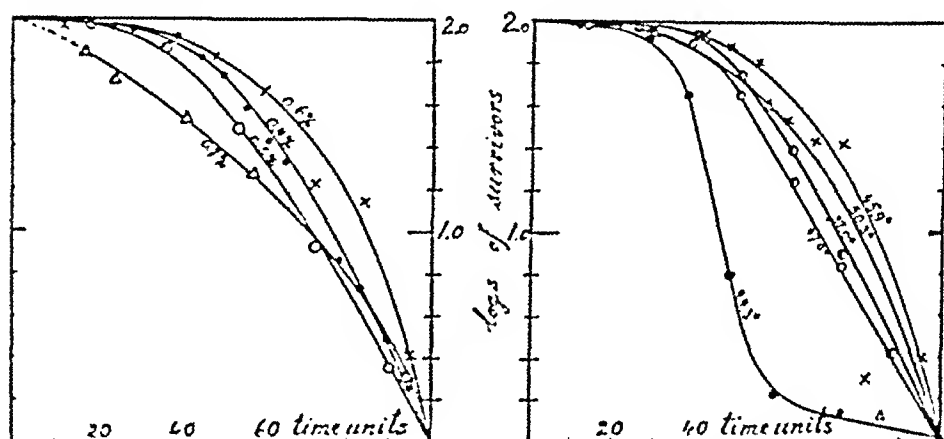


FIG. 2. Order of death of spores of *Botrytis cinerea*, on standard scale. Left: death by different concentrations of phenol; right: death by different temperatures.

Of the molds, only the spores are fit objects for a study of the order of death. We owe to J. Henderson Smith a very thorough investigation on death of spores of *Botrytis cinerea* through phenol (1921) and through heat (1923). The results of most of these experiments are presented in the two sets of curves of Fig. 2, drawn as logarithmic survivor curves to standard scale.

Smith calls attention to the observation that with increasing phenol concentration, the survivor curve approaches the logarithmic order. He also compares, in his Fig. 7, two different concentrations on standard scale. From the Fig. 2 above, it seems, however, that only the curve for 0.7 per cent phenol shows a relatively short period of no deaths while the other concentrations are fairly uniform, and show no approach. Nor do the curves on death by heat show any indication of typical change by increase of temperature.

The period of no deaths is very large in both sets of data, and suggests a fairly large number of reacting molecules. It must be kept in mind, however, that individual variation may influence survivor curves

TABLE II  
*Result of Tossing Ten Coins 1152 Times*

| Heads | Number of occurrences | Number of chances left | $\frac{1}{t} \log \frac{a}{b}$ |
|-------|-----------------------|------------------------|--------------------------------|
|       |                       | <i>per cent</i>        |                                |
| 0     | 0                     | 1152 = 100             | —                              |
| 1     | 15                    | 1137 98.7              | 0.006                          |
| 2     | 54                    | 1083 94.1              | 0.013                          |
| 3     | 138                   | 945 82.0               | 0.029                          |
| 4     | 238                   | 707 61.4               | 0.053                          |
| 5     | 269                   | 438 36.1               | 0.084                          |
| 6     | 234                   | 204 17.7               | 0.125                          |
| 7     | 134                   | 70 6.1                 | 0.174                          |
| 8     | 53                    | 17 1.48                | 0.229                          |
| 9     | 14                    | 3 0.026                | 0.298                          |
| 10    | 3                     | 0 0                    | —                              |

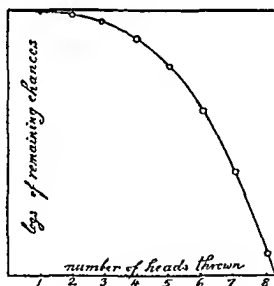


FIG. 3. A curve of chance, on standard scale. Percentage of chances to throw more than 1, 2, 3, etc. heads with 10 coins.

quite decidedly. There must have been a very wide range of variation of resistance in these experiments. Of the untreated spores used in the first phenol experiment, 8.5 per cent did not germinate even

without phenol. From these spores, too weak to develop at all, to those which resisted phenol for 2 hours, there is such a great range of variation that this might have determined the type of survivor curve.

TABLE III  
*Death of Chlamydomonas in 0.009 Per Cent HCl*

| Time of exposure | Survivors | Deaths per 5 minutes | $\frac{1}{t} \log \frac{a}{b}$ |
|------------------|-----------|----------------------|--------------------------------|
| <i>min.</i>      |           |                      |                                |
| 0                | 113       | —                    | —                              |
| 5                | 67        | 46                   | 0.045                          |
| 10               | 30        | 27                   | 0.070                          |
| 15               | 14        | 16                   | 0.066                          |
| 20               | 6         | 8                    | 0.074                          |
| 25               | 3         | 3                    | 0.060                          |
| 30               | 0         | 3                    | —                              |
| 35               | 0         | 0                    | —                              |

TABLE IV  
*Death of Colpidium by HgCl<sub>2</sub>*

| Experiment I     |                 |                      |                                | Experiment II    |                 |                 |                                |
|------------------|-----------------|----------------------|--------------------------------|------------------|-----------------|-----------------|--------------------------------|
| Time of exposure | Survivors       | Deaths per 5 minutes | $\frac{1}{t} \log \frac{a}{b}$ | Time of exposure | Survivors       | Deaths per min. | $\frac{1}{t} \log \frac{a}{b}$ |
| <i>min.</i>      | <i>per cent</i> |                      |                                | <i>min.</i>      | <i>per cent</i> |                 |                                |
| 5                | 96.96           | 3.04                 | 0.0027                         | 1                | 99.13           | 0.87            | 0.0038                         |
| 10               | 94.58           | 2.42                 | 0.0024                         | 3                | 78.9            | 10.11           | 0.0313                         |
| 15               | 66.8            | 27.78                | 0.0117                         | 5                | 46.2            | 16.35           | 0.0671                         |
| 20               | 48.9            | 17.9                 | 0.0155                         | 6                | 41.3            | 5.9             | 0.0640                         |
| 25               | 29.2            | 19.7                 | 0.0215                         | 7                | 24.1            | 17.2            | 0.0883                         |
| 30               | 24.0            | 5.2                  | 0.0266                         | 8                | 16.8            | 7.3             | 0.0969                         |
| 35               | 13.1            | 9.9                  | 0.0252                         | 11               | 0.3             | 5.5             | 0.1138                         |
| 45               | 4.0             | 3.5                  | 0.0291                         |                  |                 |                 |                                |

As an illustration, a pure chance experiment may be compared. Ten coins were flipped simultaneously 1152 times, and Table II shows how often 1, 2, 3, etc., heads occurred. The number of chances to throw more than 1, 2, 3, etc., heads corresponds to the survivors in biological experiments. The curve for the number of chances is drawn in Fig. 3 on standard scale. This curve is absolutely identical with the

above survivor curves of the mold spores. Smith concluded from his data that the order of death was entirely a chance curve. Since the calculated curve for the order of death approaches more and more the above type of chance curve as the number of reacting molecules increases, it appears rather hopeless to separate the one from the other.

With green algae, at least one good experiment is available on the death of *Chlamydomonas* in hydrochloric acid by Harvey (1909). The dead individuals were determined by the loss of motility and the dropping down to the bottom. This is again a new definition of death.

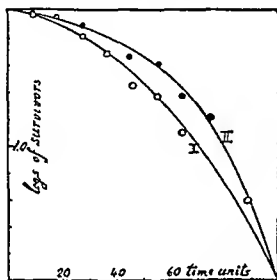


FIG. 4. Order of death of *Colpidium*. Two experiments on standard scale.

Table III shows that the death-rate is fairly constant, and that the deaths per unit time are highest in the first time interval. Harvey himself showed also that there is a straight line relation of the logarithms of survivors plotted against time.

The two experiments of Peters (1920) on the death of the flagellate *Colpidium* show just as plainly that the logarithmic order does not fit this case. The death-rate increases continuously, and the deaths per unit time show an increase followed by a decrease. An organism was considered dead when it fell to the bottom of the drop.

In order to plot these data on our standard scale,<sup>6</sup> it would be necessary to find by extrapolation the time required to kill 999 out of 1000

<sup>6</sup> Rahn, Otto, *J. Gen. Physiol.*, 1929-30, 13, 395.

individuals. This is hardly possible with the few data available and the small numbers of individuals tested. It was, therefore, necessary to change the standard time to the number of minutes necessary to kill 99 per cent of all individuals, instead of 99.9 per cent. This standard is carried through this entire paper because with the large organisms discussed here, we rarely have more than 100 individuals in one experiment.

Fig. 4 shows that more than one molecule is involved in this reaction. This curve cannot be accounted for by clumping of individuals, as each individual was counted as such under the microscope. If we wish to

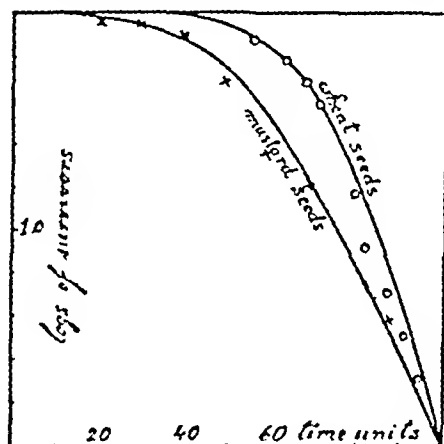


FIG. 5. Order of death of seeds, on standard scale. Wheat seeds, killed by heat, and mustard seeds, killed by bichloride of mercury.

No data could be found on the order of death of eggs.

Among the experiments with multicellular organisms, the best experiments are probably those with seeds because the test of germination is quite a severe test for the vitality of the organism, and germination could not progress very far, probably, if the vital cells of the seed had been injured beyond recovery.

The discussion might begin with the experiment on mustard seeds by Hewlett (1909) treated with  $\text{HgCl}_2$  in exactly the same way that bacteriologists treat anthrax spores. The death-rate has been shown<sup>7</sup> not to be constant, but to increase from 0.0018 to 0.0117. The survivor curve on the standard scale is shown in Fig. 5.

TABLE V  
*Death of Wheat Seeds Heated to 87.5°C.*

| Time        | Survivors | Deaths per min. | $\frac{1}{t} \log \frac{a}{b}$ |
|-------------|-----------|-----------------|--------------------------------|
| <i>min.</i> |           |                 |                                |
| 0           | 98        | 0               | —                              |
| 7           | 74        | 3               | 0.017                          |
| 8           | 60        | 14              | 0.027                          |
| 9           | 38        | 22              | 0.046                          |
| 10          | 11        | 27              | 0.095                          |
| 11          | 5         | 6               | 0.117                          |
| 12          | 2         | 3               | 0.141                          |
| 13          | 0         | 2               | —                              |

The same figure shows also the death of dry wheat seeds at a temperature of 87.5°C., as measured by Groves (1917) whose data are reproduced in Table V. In this experiment, "seeds were considered normally germinated when both root and stem had broken through the seed coat. Injury is very plainly shown by a delay in germination which is at longest exposures as much as 16 days later than the normal germination." This definition of death agrees quite well with that of the bacteriologist.

Porodko (1926) made a number of careful experiments on the death of wheat seeds steeped in hot water for various lengths of time. Later, Porodko (1927) gave a formula for the order of death in which he

<sup>7</sup> Rahn, Otto, *J. Gen. Physiol.*, 1929-30, 13, 181.



assumes that the period of no deaths is merely due to the slow penetration of heat into the seed, and that after the heat is uniform throughout the seeds, they die in logarithmic order. He proves this graphically by showing that the logarithms of survivors lie on a straight line after the period of no deaths is passed. If his curves are compared with those given by the writer<sup>2,4</sup> for more than one reacting molecule it will be seen that their lower end deviates but little from the straight line. The data at hand are not sufficient to prove the deviation. But Porodko's explanation is improbable because he would have to assume that the heat penetration requires

|               |               |
|---------------|---------------|
| at 60.4°..... | 30 seconds    |
| at 55.0°..... | 3-4 minutes   |
| at 50.0°..... | 10-15 minutes |
| at 45.0°..... | 6 hours       |

This is very improbable. Nor does Porodko give any explanation as to why the order of death should be logarithmic. His data fit the logarithmic curve, but they also fit the curve for several reacting molecules which is by far more probable.

Unless some mathematical method should be found to distinguish the number of "reacting cells" as well as the number of "reacting molecules," the results for multicellular organisms cannot be fully interpreted. In those higher organisms where the destruction of *one* vital molecule causes the death of the cell, the number of cells is identical with the number of reacting molecules. But we have no means of determining which organisms, if any, have such cells with only one reacting molecule, and our interpretation must necessarily remain rather vague.

Some animal experiments will be mentioned, however, to call attention to a certain confusion of analogies.

The death of blood corpuscles has interested biologists for some time, and Brooks (1919) quotes quite a number of investigators who studied the order of death of blood corpuscles by ultra-violet light and similar rays.

The corpuscles appear normal immediately after irradiation, but they dissolve within a few hours. All investigators, without exception, have irradiated for a definite length of time, and have observed in what order these blood cells then disappear, dissolve, "die."

This is something altogether different from the order of death we have been discussing so far, and cannot be compared at all. The time of observation is here confused with the time of exposure. In all previous experiments, organisms were exposed for several different lengths of time, and the number killed through each of these exposures was measured. It was not determined how soon after the exposure they really died; it does not matter whether the bacteria plates are counted 2 days or 7 days after exposure. But the blood corpuscles were all exposed for the same length of time and, in all published

TABLE VI  
*Observed Course of Hemolysis after Brooks, 1919*

| 10 minutes exposure to ultra-violet light |                                    | Serum hemolysis           |                       |                 |
|---|------------------------------------|---------------------------|-----------------------|-----------------|
| Time after radiation                      | Colorimetric estimate of hemolysis | Time after serum addition | Colorimetric estimate | Cell count      |
| <i>min.</i>                               | <i>per cent</i>                    | <i>min.</i>               | <i>per cent</i>       | <i>per cent</i> |
| 5   | 1                                  | 6                         | 5                     | 12              |
| 12  | 2                                  | 14                        | 21                    | 45              |
| 19  | 3                                  | 28                        | 56                    | 55              |
| 37  | 5                                  | 41                        | 67                    | 73              |
| 55  | 12                                 | 85                        | 81                    | 79              |
| 88  | 29                                 | 155                       | 82                    | 82              |
| 145                                       | 55                                 | 1320                      | 100                   | 100             |
| 205                                       | 72                                 |                           |                       |                 |
| 295                                       | 87                                 |                           |                       |                 |
| 660                                       | 100                                |                           |                       |                 |

experiments, sufficiently long to be killed. Those corpuscles were all dead when exposure ceased. The investigators only lacked the means to see it. The dissolution which was observed later is not death; it is a really immaterial secondary reaction following the destruction of the vital molecules.

Brooks (1919) in his discussion of the order of death of red blood corpuscles, mentions the two types of experiments in the same table (see Table VI).

The first experiment shows no deaths immediately after the radiation, but the erythrocytes begin to disintegrate one hour after the cause of death has been removed. The order of disintegration is a

variability curve; nothing else could be expected from organisms all of which receive exactly the same treatment of 10 minutes radiation.

In the second experiment, the blood corpuscles are continuously exposed to hemolysis. It would be comparable to bacteriological technique had we not learned from the first experiment that it takes in the average 145 minutes for a blood corpuscle to dissolve after it is doomed to die.

In order to bring out the difference more plainly, the author, together with Mr. J. A. Woerz, made some similar experiments varying the time of exposure as well as the time of observation. Chicken blood, diluted 1:10, was exposed to ultra-violet light in very shallow

TABLE VII  
*Hemolysis of Chicken Erythrocytes by Ultra-Violet Light*

| Time of exposure<br>min. | Percentage of survivors after hours |      |      |      |      |      |      |      |      |
|--------------------------|-------------------------------------|------|------|------|------|------|------|------|------|
|                          | 0                                   | 2    | 3.5  | 4.5  | 5.5  | 8    | 10   | 12.5 | 22   |
| 0                        | 98.7                                | —    | 98.7 | —    | 99.2 | 98.7 | 98.7 | 97.0 | 97.1 |
| 1                        | 98.7                                | —    | 98.4 | —    | —    | 98.1 | 97.7 | 97.7 | 95.5 |
| 2                        | 99.3                                | —    | 98.9 | —    | 99.3 | 98.2 | 98.2 | 95.6 | 80.8 |
| 4                        | 99.6                                | 99.6 | 99.6 | 99.6 | 99.6 | 99.6 | 97.9 | 89.8 | 57.2 |
| 8                        | 99.0                                | 99.0 | 98.6 | 99.0 | —    | 83.0 | 61.3 | 42.3 | 11.6 |
| 16                       | 100.0                               | 96.3 | 86.0 | 58.6 | 48.0 | 33.0 | 23.7 | 11.2 | 2.4  |

dishes and then sealed with vaseline in hemacytometers, incubated at 37°, and counted at intervals. The count after 22 hours was taken as final, and corpuscles not dissolved within 22 hours after exposure were considered unharmed, or "survivors." Table VII shows all essential data.

Fig. 6 presents these data in two different ways. The figure on the left shows the interpretation used by Brooks and all other students of the "order of death" of blood corpuscles. The curves of the figure on the right correspond to bacteriological survivor curves, and only the lowest curve, after 22 hours of observation, shows how many blood cells were really killed by exposure. This curve is not a straight line. It has a period of "no deaths." More than one molecule in the cells

must be injured by the rays before the cell dissolves. To judge from the relatively very short period of "no deaths," the number of reacting molecules must be quite small.

This confusion of time of observation and time of exposure is not limited to experiments with blood corpuscles. The experiments with

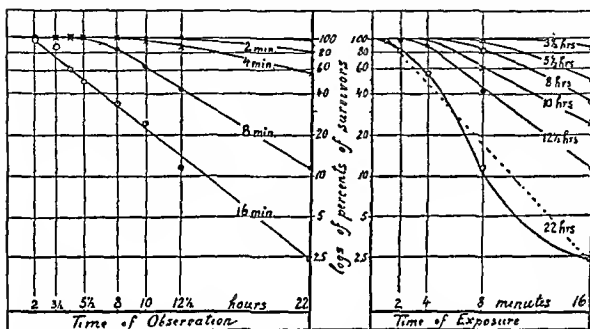


FIG. 6. Order of death of erythrocytes. The same data plotted in two different ways.

TABLE VIII  
Death of *Tribolium confusum*  
Percentage of beetles dying from x-ray radiation

|                      |   |   |     |     |     |     |    |    |     |    |    |     |
|----------------------|---|---|-----|-----|-----|-----|----|----|-----|----|----|-----|
| No. of days.....     | 1 | 2 | 3.2 | 4.2 | 4.8 | 5.2 | 6  | 7  | 7.2 |    |    |     |
| Percentage dead..... | 0 | 2 | 2   | 25  | 60  | 72  | 94 | 99 | 100 |    |    |     |
| No. of days.....     | 1 | 2 | 3   | 4   | 5   | 6   | 7  | 8  | 8.3 | 9  | 10 | 11  |
| Percentage dead..... | 0 | 2 | 2   | 3   | 7   | 28  | 57 | 83 | 91  | 97 | 99 | 100 |

the influence of x-rays upon the beetle *Tribolium confusum* by Davey (1917) are another such example. Davey exposed 100 beetles to x-rays for a certain length of time (which did not kill them immediately) and then counted the number of dead individuals day after day. The two sets of figures in Table VIII are the only data given

by him. In the controls, never more than 4 per cent died within the first 15 days.

TABLE IX

*Percentage Survivors of Tomato Moth Larvae on Plants Sprayed with Arsenate of Lead*

| Survivors after | Pounds of lead arsenate (20 per cent $As_2O_3$ ) per 100 gallons |          |          |          |          |          |
|-----------------|--|----------|----------|----------|----------|----------|
|                 | 2  | 3        | 4        | 5        | 6        | 10       |
| days            | per cent   | per cent | per cent | per cent | per cent | per cent |
| 1               | 97.1   | 94.6     | 90.2     | 82.9     | 84.7     | 85.3     |
| 2               | 82.4   | 67.2     | 71.5     | 47.2     | 49.3     | 54.6     |
| 3               | 61.8   | 46.0     | 37.3     | 19.3     | 28.4     | 22.6     |
| 4               | 47.1   | 19.5     | 20.6     | 8.4      | 12.1     | 11.9     |
| 5               | 35.4   | 13.3     | 8.5      | 6.6      | 5.8      | 3.9      |
| 6               | 29.5   | 9.8      | 5.2      | 4.8      | 2.3      | 2.6      |
| 7               | 26.6   | 7.1      | 3.9      | 3.6      | 1.7      | 0        |
| 8               | 17.7   | 3.6      | 1.9      | 3.0      | 0.6      | 0        |
| 9               | 14.8   | 3.6      | 0.6      | 2.4      | 0        |          |
| 10              | 11.9   | 1.8      | 0        | 1.2      |          |          |
| 11              | 6.0  | 0.9      |          | 0        |          |          |
| 12              | 6.0  | 0.9      |          |          |          |          |
| 13              | 3.1  | 0        |          |          |          |          |
| 14              | 0  |          |          |          |          |          |

TABLE X

*Tadpoles Killed by Exposure to 35.5°C.*

| Time | Total number | Killed | Percentage of survivors |
|------|--------------|--------|-------------------------|
| min. |              |        |                         |
| 0    |              |        | 100                     |
| 5    | 200          | 0      | 100                     |
| 10   | 201          | 11     | 95                      |
| 12.5 | 238          | 121    | 49                      |
| 15   | 222          | 200    | 10                      |
| 20   | 203          | 199    | 2                       |
| 25   | 116          | 116    | 0                       |
| 30   | 97           | 95     | 2                       |

If the beetles had been counted by the bacteriological technique, they would all have been counted as dead at the end of the radiation. None of the radiated beetles multiplied; they all had a shortened ex-

pectation of life. We must assume from the data that some essential molecules in the vital cells of all the beetles had been destroyed, but the rest of the cells, less sensitive than those few vital ones, survived and performed their routine tasks in the ordinary way just as yeast cells may continue fermenting after they have lost the capacity to form colonies. The beetles were all doomed, yet gave the impression of normal healthy individuals until their premature death proved the contrary.

As examples of experiments showing an order of death in the customary sense of the word, there are quoted in Tables IX and X the killing

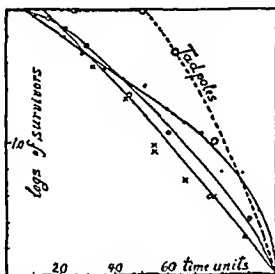


FIG. 7. Order of death by heat of tadpoles (dotted line) and of tomato moth larvae by three different concentrations of arsenate of lead. (standard scale).

of tomato moth larvae by different concentrations of arsenate of lead, from Lloyd, (1920) and the killing of tadpoles (which were nearly ready to become frogs) by heat (35.5°C.) according to Boycott (1920). Fig. 7 shows the logarithmic survivor curves. They indicate plainly that a considerable number of molecules must be inactivated before these organisms are dead, and there is a suggestion that this number is larger in the tadpoles than in the moth larvae. This statement cannot be made more positive because we have to consider the variation of individuals to resistance which was probably greater in the moth larvae than in the tadpoles. This variation causes the period of no deaths to change very gradually into the death curve while the change is quite abrupt if all individuals have the same resistance.<sup>2</sup>

There is a possibility of determining the number of molecules whose inactivation is sufficient to cause death, provided that we can eliminate variation in resistance. In this case, the period of no deaths is plainly marked. For any rate of death and for any number of reacting molecules  $r$ , the percentage of survivors can be computed from the formula developed. Table XI shows such computation for the case of two reacting molecules. The formula for the dead individuals at the time  $n$  in this case is

$$D_n = 100 (1 - q^n - nq^{n-1}p)$$

TABLE XI  
*Theoretical Percentage of Survivors for  $r = 2$  Reacting Molecules*

| Time $n$ | $q = 0.1$ | $q = 0.2$ | $q = 0.3$ | $q = 0.4$ | $q = 0.5$ | $q = 0.6$ | $q = 0.7$ | $q = 0.8$ | $q = 0.9$ |
|----------|-----------|-----------|-----------|-----------|-----------|-----------|-----------|-----------|-----------|
| 1        | 100       | 100       | 100       | 100       | 100       | 100       | 100       | 100       | 100       |
| 2        | 19        | 36        | 51        | 64        | 75        | 84        | 91        | 96        | 99        |
| 3        | 3.81      | 10.4      | 21.6      | 35.2      | 50        | 64.8      | 78.4      | 89.6      | 97.2      |
| 4        | 0.37      | 2.72      | 8.37      | 17.92     | 31.21     | 47.5      | 65.2      | 82.6      | 94.8      |
| 5        | 0.06      | 0.77      | 2.96      | 8.70      | 18.7      | 33.7      | 52.8      | 73.3      | 91.9      |
| 6        | 0         | 0         | 1.33      | 4.09      | 10.9      | 23.3      | 42.0      | 65.4      | 88.8      |
| 7        | 0         | 0         | 0.43      | 1.89      | 6.2       | 15.9      | 32.9      | 57.2      | 85.0      |
| 8        | 0         | 0         | 0         | 0.85      | 3.6       | 10.6      | 25.5      | 49.9      | 81.2      |
| 9        | 0         | 0         | 0         | 0         | 1.95      | 7.1       | 19.6      | 43.3      | 77.5      |
| 10       | 0         | 0         | 0         | 0         | 1.07      | 4.6       | 14.9      | 37.3      | 73.5      |
| 11       | 0         | 0         | 0         | 0         | 0.59      | 3.0       | 11.3      | 32.0      | 69.7      |

where  $p$  is the proportion of molecules acted upon in unit time, and  $q = 1 - p$ . From this, we obtain the percentage of survivors

$$S = 100 (n - q(n - 1)) q^{n-1}$$

The period of no deaths in this case lasts just one time unit. If the period of no deaths in any experiment can be accurately measured, the other data can be compared with Table XI; if they fit into any of these sets of numbers, death was caused by inactivation of two molecules per organism.

The data on *Colpidium* by Peters (Table IV) may serve as an example. The period of no deaths in Experiment II is practically one minute. The data for the later times fit fairly well between the values

of Table XI computed for  $q = 0.6$  and  $q = 0.7$ , as may be seen by the following comparison:

Percentage of Survivors

| $n =$                               | 1     | 3    | 5    | 6    | 7    | 8    | 11   |
|-------------------------------------|-------|------|------|------|------|------|------|
| Computed for $q = 0.6$ .....        | 100   | 64.8 | 33.7 | 23.3 | 15.9 | 10.6 | 3.0  |
| Observed for <i>Colpidium</i> ..... | 99.13 | 78.9 | 46.2 | 41.3 | 24.1 | 16.8 | 0.3  |
| Computed for $q = 0.7$ .....        | 100   | 78.4 | 52.8 | 52.8 | 32.9 | 25.5 | 11.3 |

The agreement is quite good except for the last value which might be brought about by variation in resistance.

In Experiment I, a graphical treatment of the data indicates the period of no deaths to be about 8 minutes. We must also determine graphically the survivors for 16 minutes ( $n = 2$ ), 24 minutes ( $n = 3$ ) etc. The results check fairly well for  $q = 0.4$ .

Percentage of Survivors

| $n =$                               | 1    | 2    | 3    | 4     | 6   |
|-------------------------------------|------|------|------|-------|-----|
| minutes                             | 8    | 16   | 24   | 30-35 | 48  |
| Computed for $q = 0.4$ .....        | 100  | 64.0 | 35.2 | 17.9  | 4.1 |
| Observed for <i>Colpidium</i> ..... | 95.8 | 64   | 33   | 17    | 4.0 |

Again, the agreement is quite satisfactory, and we might draw the conclusion that death of *Colpidium* (i.e., cessation of motion) is caused by the inactivation of two molecules.

A fair agreement is also found for the death of blood corpuscles. The data from Table VII observed after 22 hours coincide approximately with the computation for  $q = 0.6$ . Fig. 6 shows that the period of no deaths is practically one minute.

Percentage of Survivors

| $n =$                               | 1    | 2    | 4    | 8    | 16   |
|-------------------------------------|------|------|------|------|------|
| Computed for $q = 0.6$ .....        | 100  | 84   | 47.5 | 10.6 | 0.33 |
| Observed with blood corpuscles..... | 95.5 | 80.8 | 57.2 | 11.6 | 2.4  |

The data fluctuate a great deal, as may also be seen from Fig. 6, but there is a general agreement, except again for the last value.



Quite different is the case with fruit flies. The period of no deaths is between 17 and 20 minutes. If two reacting molecules would satisfy the order of death, 17–20 minutes should be the unit of time,  $n$ . We obtain the survivor data graphically (Table XII).

The values for  $q$  estimated from Table XI are decreasing rapidly with time; even if we chose the time unit as 25 minutes,  $q$  would decrease. A decrease of  $q$  means an increase of the death-rate. This increase indicates, that the number of reacting molecules is larger than 2, and, since the increase of the death rate is rapid, the number of reacting molecules is much larger than 2. Nothing else could be expected of a metazoon.

TABLE XII  
*Death of Fruit Flies*

| $n \approx$ | Time unit = 17.5 minutes |                         |                             | Time unit = 20 minutes |                         |                             |
|-------------|--------------------------|-------------------------|-----------------------------|------------------------|-------------------------|-----------------------------|
|             | Minutes                  | Survivors, experimental | Corresponding value for $q$ | Minutes                | Survivors, experimental | Corresponding value for $q$ |
| 1           | 17.5                     | 100                     | —                           | 20                     | 100                     | —                           |
| 2           | 35.0                     | 65.4                    | 0.4                         | 40                     | 45.5                    | 0.26                        |
| 3           | 52.5                     | 14.5                    | 0.23                        | 60                     | 8.8                     | 0.18                        |
| 4           | 70.0                     | 1.1                     | 0.14                        | 80                     | 0.3                     | 0.09                        |

In order to actually determine the number of reacting molecules in this way, it would not only be necessary to have a more accurate formula, but also to have data on individuals of uniform resistance. This might be accomplished to a certain degree with small organisms, or with seeds from carefully bred plants. But the author knows of no data where an effort has been made to eliminate variability. With an increasing number of reacting molecules, the calculated data will be quite close to each other, and considering the large experimental error, the actual number of reacting molecules could be only approximately estimated by this method.

The small number of such vital molecules in unicellular organisms makes it appear probable that in larger organisms also, the number of such molecules per cell is not very large. This would mean that the reactions of these molecules in each cell do not follow the mass law, but the law of chance. Even with one hundred reacting molecules per

cell, not all cells die at the same time; after a period of no deaths of one hundred time units, 5 per cent die between 100–105 units, 53 per cent between 105–110 units, 38 per cent between 110–115 units, and 4 per cent between 115–120 units.<sup>8</sup>

There must be a lack of continuity of reaction owing to the limited number of molecules of each kind, comparable to the quantum theory in energetics, which is also based upon certain limits in the division of matter. The final reaction in each cell, under these circumstances, will be identical, but the reaction in each of a number of identical cells may take place at different times.

It seems possible to imagine that this lack of continuity and the improbability of similar cells reacting alike at the same time is of greater biological significance. It might be quite an essential factor in causing dissimilarity of individuals of the same species, of leaves on the same tree, of identical twins, etc.

#### SUMMARY

In a previous paper it has been shown that the logarithmic order of death of bacteria can be accounted for by the assumption of some very unstable molecules so essential for reproduction that the inactivation of only one such molecule per cell prevents reproduction and makes the cell appear "dead" according to the standard method of counting living bacteria.

In the present paper dealing with the order of death of larger organisms, only a motile alga, *Chlamydomonas*, is shown to have the same order of death.

The very scant material on the order of death of yeasts is contradictory. It seems possible that more than one molecule must be destroyed to kill a yeast cell.

With the spores of a mold, *Botrytis cinerea*, the number of "reacting molecules" is decidedly larger than 1.

A flagellate, *Colpidium*, gave a survivor curve suggesting the destruction of two molecules before motility ceases. Erythrocytes exposed to ultra-violet light also follow the formula for two reacting molecules.

<sup>8</sup> Rahn, Otto, *J. Gen. Physiol.*, 1929, 13, 197.

The analysis of the survivor curves of multicellular organisms is not possible because no distinction between the number of essential molecules and the number of essential cells seems possible. Besides, variability of resistance changes the shape of the survivor curves in such a way that it becomes impossible to differentiate between variability and actual survivor curve.

The general results seem to justify the assumption that in each cell, the number of molecules which are really essential for life and reproduction, is quite limited, and that, therefore, equal cells will not react simultaneously, though ultimately the reaction will be the same.

This theory of lack of continuity in cell reactions owing to the limited number of reacting molecules is an analogon to the quantum theory where continuity ceases because division of matter reaches a limit.

It seems probable that this lack of continuity in cell reactions has a general biological significance reaching beyond the order of death.

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# A METHOD FOR DETERMINING THE OXYGEN CONSUMPTION OF A SINGLE CELL

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## I

### INTRODUCTION

Several methods are available for determining the  $O_2$  consumption of a large number of cells. Only one author, Kalmus (1), has attempted to estimate the metabolism of a single cell directly. His method, as he applied it to *Paramecium* is briefly as follows: An animal in a small amount of culture medium is drawn up into a capillary tube. This is followed by a little paraffin oil and next a column of air. This end of the tube is now sealed in a flame. KOH 10 per cent and paraffin oil are drawn in, in that order, through the opposite end of the tube, by gently warming the enclosed air column and allowing it to contract subsequently. After the capillary has cooled, the movement of the meniscus between the oil and the KOH is followed by means of an ocular micrometer. Readings are made every 10 minutes for one hour. From these the contraction of the trapped air is determined, the decrease in volume representing the amount of oxygen consumed by the *Paramecium*. The method was later modified (2) slightly so that the animal was located in a bulb at one end of the capillary, but the essentials were the same as before.

Reasonably constant results were obtained on repetition of Kalmus' work (Table I). It later appeared, however, that the results were equally constant whether or not an animal were present. When capillary tubes of about the same diameter are heated in the same manner and treated similarly throughout the course of the procedure, they will cool down at the same rate for the corresponding hour, over

a period of many hours. This is illustrated in Table II. A capillary containing no animal was observed for 7 hours after a preliminary cooling in water for one half hour following the fusing of one end. Since the motion of the meniscus continued for at least 7 hours it seemed essential to devise a method which did not entail the application of heat to the capillary tube.

TABLE I

*Apparent O<sub>2</sub> Consumption of a Single Paramecium by Method of Kalmus*

| Experiment | Apparent O <sub>2</sub> consumption in mm. <sup>3</sup> per hour |
|------------|--|
| 1          | 0.045  |
| 2          | 0.024  |
| 3          | 0.033  |
| 4          | 0.043  |
| 5          | 0.042  |
| 6          | 0.052  |
| 7          | 0.042  |

TABLE II

*Motion of Meniscus in a Kalmus Tube Containing No Animal*  
Each unit is equivalent to a decrease in volume of 0.009 mm.<sup>3</sup>

| Elapsed time in hours | Total motion of meniscus in units |
|-----------------------|-----------------------------------|
| 1                     | 4.5                               |
| 2                     | 7.5                               |
| 3                     | 10.0                              |
| 4                     | 12.0                              |
| 7                     | 14.5                              |

## II

*Apparatus and Method of Calculation*

The respirometer consists of a glass capillary tube about 6 cm. in length and 0.3 mm. internal diameter. The diameter may be varied to suit the size of the cell under investigation. It is desirable to have the capillary of as small diameter as possible without crowding the cell. This increases the sensitivity of the respirometer since it permits maximum horizontal readings as the air column decreases.

The tube is filled by means of the injection system of a Chambers' micromanipulator. The materials are drawn in, in such order as to give the final arrangement indicated in Fig. 1. First, mineral oil, followed by the cell in a small amount of culture medium; next, air; then a small amount of 5 per cent KOH, and finally mineral oil again. Approximate dimensions of each column are shown in the diagram. It is well to have a minimum of culture medium in order to make the system as compact as possible. On the other hand, it is essential to have a moderate excess of air so that the oxygen consumption of the cell will not lower the partial pressure of the oxygen too markedly.

Once filled, the tubes are placed in a water bath so constructed that it may be held firmly by the mechanical stage of a microscope. The tubes are arranged so that oil extends out to both ends, thus diminishing the ease of access of  $O_2$ . They are held by means of rubber bands in two parallel grooved ledges cemented along the base of the

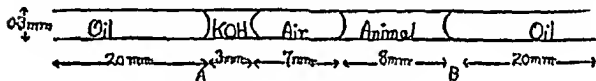


FIG. 1. Microrespirometer

bath. The direction of the capillary is such that it is parallel to the horizontal movement of the mechanical stage. A series of six or eight tubes will conveniently fit in the bath and with each group a control capillary must be run. This latter is made in a manner identical with the experimental tubes, except that no cell is included in the culture medium.

The water bath and microscope are now placed in a constant temperature air chamber. Allowing about a half hour for the system to reach thermal equilibrium, the first readings are then taken. Since it is possible to get much sharper readings at the oil-liquid meniscus than at that between air and liquid, the distance between the two oil menisci is recorded, (A-B). For all practical purposes, however, what is being followed is the change in volume of the enclosed air since that is the only variable directly measured during the course of the experiment. At the same time the internal diameter of the tube is recorded by means of a calibrated micrometer scale in the ocular



lens of the microscope. The readings on the mechanical stage can be taken to hundredths of a millimeter by interpolating on the vernier which is graduated to tenths. The approximate length of the air columns should be noted for later reference concerning thermal and barometric corrections.

The duration of an experiment varies with the oxygen consumption of the cell being investigated. In general, it is advisable to allow a minimum time necessary to give a change in length of the air column equal to 0.4 mm. Under these conditions, the possible error from reading will not exceed 10 per cent.

To illustrate this discussion, data and calculations from a single experiment are given. The cell under investigation was *Actinosphaerium cichhornii*.

|                        | Experimental |            |         |                  | Control    |         |                  |
|------------------------|--------------|------------|---------|------------------|------------|---------|------------------|
|                        | Time         | A (mm.)    | B (mm.) | Difference (mm.) | A (mm.)    | B (mm.) | Difference (mm.) |
| 1930                   |              |            |         |                  |            |         |                  |
| Aug. 23                | 9:20 a.m.    | 55.15      | 77.68   | 22.53            | 72.84      | 56.23   | 16.61            |
|                        | 2:20 p.m.    | 55.21      | 77.72   | 22.51            | 72.89      | 56.26   | 16.63            |
|                        | 5:35 p.m.    | 55.25      | 77.72   | 22.47            | 72.91      | 56.26   | 16.65            |
| Aug. 24                | 8:30 a.m.    | 55.47      | 77.69   | 22.22            | 72.93      | 56.37   | 16.56            |
| Experimental           |              |            |         |                  | Control    |         |                  |
| Diameter tube.....     |              | 21.5 units |         |                  | 20.5 units |         |                  |
| Length air column..... |              | 6.3 mm.    |         |                  | 7.35 mm.   |         |                  |

In 23.2 hours the air column in the experimental tube decreased 0.31 mm. in length. The control column decreased 0.05 mm. From these figures and the volumes of the respective air columns the thermobarometric correction is -0.05 mm. The corrected shortening of the air column was 0.26 mm. The diameter of the tube was 21.5 units on the micrometer scale. (1 unit = 0.0177 mm.) Thence the  $O_2$  consumed =  $\frac{0.26 \times 0.1138}{23.2} = 0.00128 \text{ mm.}^3$  per hour, where 0.1138 mm.<sup>2</sup> is the cross-sectional area of the capillary with a diameter 0.38 mm.

The control also corrects for any volume changes referable to the differing osmotic pressures of the KOH and the culture medium with a consequent water vapor exchange. This is proved by running several control tubes simultaneously and finding identical corrections from them.

## III

*Discussion of Errors*

In this method there are several possible sources of error which must be considered. The diffusion of a gas through a capillary tube is slow. If there is a lag in the absorption of  $\text{CO}_2$ , the subsequent  $\text{O}_2$  consumption readings will be too small. It seems, however, that while the  $\text{CO}_2$  is probably not immediately taken up by the  $\text{KOH}$ , the system rapidly assumes a state of equilibrium, as evidenced by the linear character of the curve obtained by plotting  $\text{O}_2$  consumption against time (Fig. 2). The ease of solubility of  $\text{CO}_2$  in the culture me-

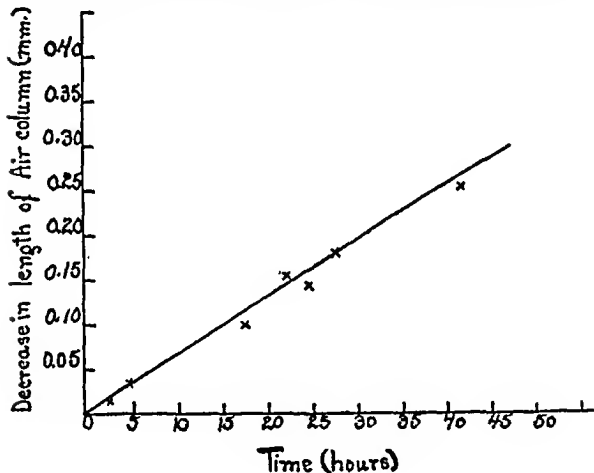


FIG. 2.  $\text{O}_2$  consumption curve

dium further promotes the quick arrival at a steady state as does the presence of a motile cell such as a *Paramecium*. For all practical purposes, then, the readings obtained are as satisfactory as if all the  $\text{CO}_2$  were immediately absorbed by the alkali.

A second question arises in connection with the possible influx of oxygen, from without, to the cell.

In earlier experiments mercury was used as the seal, but it was found that the purest mercury available at that time was toxic to the animals. Although the cells did not touch the liquid directly, the coming in contact with that part of the tube over which mercury had already been drawn was sufficient to kill an *Actinosphaerium* rapidly. At the present time further investigations are in progress using mercury distilled *in vacuo*, with the idea that impurities rather than the mercury itself were the toxic agents.

The fact that oxygen is soluble in mineral oil must also be considered. The viscosity of the oil, however, will tend to slow down gaseous diffusion through it. With that in view, the oil columns are made as long as is conveniently possible. But it is at the oil-liquid interface that the most effective barrier to gaseous diffusion exists. In order to establish the magnitude of the gaseous inflow, sets of tubes each with several cells were run. The more rapid  $O_2$  consumption of several cells together would lead to a higher gradient of  $O_2$  pressure between the air inside and outside the capillary so that there would be an increased rate of  $O_2$  inflow. With a doubled  $O_2$  consumption, for example, the rate of diffusion might be doubled. This would result in the average  $O_2$  consumption per single cell, calculated from experiments which used several cells in one tube, having a lower value than the result with the single cell. Actually such is found to be the case. The values from the multiple-cell experiments are definitely lower than those from the single-cell ones. For example, with *Paramecium caudatum*, the results for which average  $0.00049 \text{ mm.}^3$  of  $O_2$  per hour by the single-cell method, a run with three animals in a respirometer gave an average of  $0.00033 \text{ mm.}^3$  per individual. For *Actinosphaerium eichhornii*, where the  $O_2$  consumed is somewhat greater ( $0.00113 \text{ mm.}^3$  per hour by the single-cell method), the decrease with several animals is correspondingly greater; so that, with two individuals in a tube, the value per animal is half the first figure. Under constant conditions, the results are remarkably consistent, as will be shown later.

Readings on the mechanical stage are accurate to at least  $0.02 \text{ mm.}$  To check the method used for determining the volume of the tube, and especially to test the accuracy of this method in reading diameters,

the following procedure was employed. A small amount of mercury was drawn into a capillary. Its volume was determined by the method described above, and its weight calculated from the specific gravity. The mercury was then carefully poured out and weighed, and the actual weight compared with that experimentally obtained. The diameter of a capillary is most readily determined at the point where the oil meniscus approaches the internal surface of the tube tangentially. With moderate illumination, this appears as a sharp white line. Needless to say, the capillary tube should be so drawn that its internal diameter does not vary within measurable limits throughout its length. An error may be introduced by failure to correct for the refraction of light by the glass tube. This error is a function of the thickness of the capillary wall and of the difference between the refractive indices of glass and water. Since the wall is thin and the difference between the refractive indices is small (0.22), the error involved is of the second order, and negligible. The experiments involving the actual weighing of a measured volume of mercury substantiate this conclusion.

That the solutions employed may not be in equilibrium with the air at the start, is a possible source of error. For that reason the KOH solution, culture medium, and mineral oil should be exposed to the air before being used.

Large errors may be introduced if the culture medium is not sterile. For example, in an experiment with *Colpidium colpoda* grown in a medium heavy with bacteria, the culture medium was not filtered. All of these tubes, including the control, consumed an amount of oxygen far beyond reasonable limits. Whenever the control readings drop markedly in the absence of any temperature change, an error of this nature is indicated.

A simple calculation shows that the magnitude of surface tension forces at the air—culture medium and culture medium-oil interfaces is of the order of 0.1 per cent of atmospheric pressure. From this point of view, then, any surface-active substances liberated by the cell will produce negligible effects.

It might be suspected that adhesive forces would restrain the liquids in the capillary from free motion. That such is not the case is evidenced by the slight but definite shift to-and-fro of points *A* and *B* (Fig. 1) from reading to reading.

## IV

## RESULTS

The first organism investigated was *Actinosphaerium eichhornii*. The animals were all taken from a single culture. Each one was washed

TABLE III

*Rate of O<sub>2</sub> Consumption of a Single Actinosphaerium*

| Number       | Duration in hours | Mm. <sup>3</sup> of O <sub>2</sub> consumed per hour |
|--------------|-------------------|--|
| 1            | 19.00             | 0.00126  |
| 2            | 8.00              | 0.00090  |
| 3            | 23.00             | 0.00124  |
| 4            | 23.00             | 0.00124  |
| 5            | 23.00             | 0.00088  |
| 6            | 20.75             | 0.00123  |
| 7            | 20.75             | 0.00107  |
| 8            | 20.75             | 0.00104  |
| 9            | 20.75             | 0.00133  |
| Average..... |                   | 0.00113  |

Temp. range:  $21.8 \pm 1^{\circ}\text{C}$ .

TABLE IV

*Rate of O<sub>2</sub> Consumption of a Single Paramecium*

| Number       | Duration in hours | Mm. <sup>3</sup> of O <sub>2</sub> consumed per hour |
|--------------|-------------------|--|
| 1            | 21.75             | 0.00045  |
| 2            | 21.75             | 0.00050  |
| 3            | 43.75             | 0.00044  |
| 4            | 19.00             | 0.00061  |
| 5            | 43.75             | 0.00052  |
| 6            | 21.50             | 0.00044  |
| Average..... |                   | 0.00049  |

Temp. range:  $21.2 \pm 1^{\circ}\text{C}$ .

twice with double distilled water and the third wash water was used as medium for the experiment. In diameter the individuals varied slightly, averaging 0.3 mm.

The results from four successive series are incorporated in Table III. The  $O_2$  consumed per hour per animal ranged from 0.00088 mm.<sup>3</sup> to 0.00133 mm.<sup>3</sup> with an average result of 0.00113 mm.<sup>3</sup>

A series of *Paramecium caudatum* in culture medium gave the results shown in Table IV. The  $O_2$  consumed per hour per animal ranged from 0.00044 mm.<sup>3</sup> to 0.00061 mm.<sup>3</sup> with an average of 0.00049 mm.<sup>3</sup>

## V

### DISCUSSION

Considerable work has been done in the field of protozoan metabolism. Practically all of this has been of a qualitative nature so that only in a few cases are there data from which the oxygen consumption of a single protozoan can be calculated. The papers of Kalmus have already been mentioned. For *Paramecium caudatum* he found the oxygen consumption per hour per individual to be about 0.0052 mm.<sup>3</sup> In some unpublished experiments Necheles (3) using the Warburg apparatus, concluded that a single *Paramecium caudatum* used 0.00385 mm.<sup>3</sup> of  $O_2$  per hour at 19° C. Lund (4) in a study of the respiration of *Paramecium caudatum* gives several references to experiments in which he counted the actual numbers of organisms being used. From these it can be determined that a single paramecium, starved for 48 hours previous to the run, consumes 0.00004 mm.<sup>3</sup> of  $O_2$  per hour, while in one fed on dead yeast the  $O_2$  consumption increases over three-fold, i.e., to 0.00014 mm.<sup>3</sup> Zweibaum (5) with the aid of the Thunberg apparatus, followed the metabolism of *Paramecium caudatum* through its reproductive cycle. Immediately before conjugation an individual uses 0.00074 mm.<sup>3</sup> of  $O_2$  per hour. During conjugation there is a sharp increase to 0.00348 mm.<sup>3</sup> which quickly drops off to 0.00068 mm.<sup>3</sup>; the  $O_2$  consumption then gradually rising to 0.00225 mm.<sup>3</sup> within a week after conjugation. For *Paramecium aurelia*, Barratt (6) found the  $CO_2$  production of 200,000 individuals at 19–21° C. to be 1.2 mg. over a period of 24 hours. The  $CO_2$  given off by a single animal would then be 0.00012 mm.<sup>3</sup> per hour. Assuming an R. Q. between 0.7 and 1.0, the  $O_2$  consumption per hour would be of the order of 0.00015 mm.<sup>3</sup> per hour. Of course it must be remembered that the different species of *Paramecium* may show considerable metabolic variation. From the above, however, it is apparent that the hundred-fold variation of results cited in the literature makes any comparison of results unprofitable.

## IV

## RESULTS

The first organism investigated was *Actinosphaerium eichhornii*. The animals were all taken from a single culture. Each one was washed

TABLE III  
*Rate of O<sub>2</sub> Consumption of a Single Actinosphaerium*

| Number                   | Duration in hours | Mm. <sup>3</sup> of O <sub>2</sub> consumed per hour |
|--------------------------|-------------------|--|
| 1                        | 19.00             | 0.00126  |
| 2                        | 8.00              | 0.00090  |
| 3                        | 23.00             | 0.00124  |
| 4                        | 23.00             | 0.00124  |
| 5                        | 23.00             | 0.00088  |
| 6                        | 20.75             | 0.00123  |
| 7                        | 20.75             | 0.00107  |
| 8                        | 20.75             | 0.00104  |
| 9                        | 20.75             | 0.00133  |
| Average.....             |                   | 0.00113  |
| Temp. range: 21.8 ± 1°C. |                   |  |

TABLE IV  
*Rate of O<sub>2</sub> Consumption of a Single Paramecium*

| Number                   | Duration in hours | Mm. <sup>3</sup> of O <sub>2</sub> consumed per hour |
|--------------------------|-------------------|--|
| 1                        | 21.75             | 0.00045  |
| 2                        | 21.75             | 0.00050  |
| 3                        | 43.75             | 0.00044  |
| 4                        | 19.00             | 0.00061  |
| 5                        | 43.75             | 0.00052  |
| 6                        | 21.50             | 0.00044  |
| Average.....             |                   | 0.00049  |
| Temp. range: 21.2 ± 1°C. |                   |  |

twice with double distilled water and the third wash water was used as medium for the experiment. In diameter the individuals varied slightly, averaging 0.3 mm.

The results from four successive series are incorporated in Table III. The  $O_2$  consumed per hour per animal ranged from 0.00088 mm.<sup>3</sup> to 0.00133 mm.<sup>3</sup> with an average result of 0.00113 mm.<sup>3</sup>

A series of *Paramecium caudatum* in culture medium gave the results shown in Table IV. The  $O_2$  consumed per hour per animal ranged from 0.00044 mm.<sup>3</sup> to 0.00061 mm.<sup>3</sup> with an average of 0.00049 mm.<sup>3</sup>

## V

### DISCUSSION

Considerable work has been done in the field of protozoan metabolism. Practically all of this has been of a qualitative nature so that only in a few cases are there data from which the oxygen consumption of a single protozoan can be calculated. The papers of Kalmus have already been mentioned. For *Paramecium caudatum* he found the oxygen consumption per hour per individual to be about 0.0052 mm.<sup>3</sup> In some unpublished experiments Necheles (3) using the Warburg apparatus, concluded that a single *Paramecium caudatum* used 0.00385 mm.<sup>3</sup> of  $O_2$  per hour at 19° C. Lund (4) in a study of the respiration of *Paramecium caudatum* gives several references to experiments in which he counted the actual numbers of organisms being used. From these it can be determined that a single paramecium, starved for 48 hours previous to the run, consumes 0.00004 mm.<sup>3</sup> of  $O_2$  per hour, while in one fed on dead yeast the  $O_2$  consumption increases over three-fold, i.e., to 0.00014 mm.<sup>3</sup> Zweibaum (5) with the aid of the Thunberg apparatus, followed the metabolism of *Paramecium caudatum* through its reproductive cycle. Immediately before conjugation an individual uses 0.00074 mm.<sup>3</sup> of  $O_2$  per hour. During conjugation there is a sharp increase to 0.00348 mm.<sup>3</sup> which quickly drops off to 0.00068 mm.<sup>3</sup>; the  $O_2$  consumption then gradually rising to 0.00225 mm.<sup>3</sup> within a week after conjugation. For *Paramecium aurelia*, Barratt (6) found the  $CO_2$  production of 200,000 individuals at 19–21° C. to be 1.2 mg. over a period of 24 hours. The  $CO_2$  given off by a single animal would then be 0.00012 mm.<sup>3</sup> per hour. Assuming an R. Q. between 0.7 and 1.0, the  $O_2$  consumption per hour would be of the order of 0.00015 mm.<sup>3</sup> per hour. Of course it must be remembered that the different species of *Paramecium* may show considerable metabolic variation. From the above, however, it is apparent that the hundred-fold variation of results cited in the literature makes any comparison of results unprofitable.



A point of interest in our experiments was the question of whether the metabolic activity of the cells varied over the long course of the measuring period. The possibility of toxic metabolic products exerting an effect on the cell as the experiment progressed was ruled out by recording data over two successive 24 hour periods with the same cell. The  $O_2$  consumed in the second half of the run always agreed with the figure for the first half, within the 20 per cent allowed for experimental errors (Fig. 2).

By the method described it is possible to obtain data on the  $O_2$  consumption of a single cell. In view of the various factors such as physiological state, phase in reproductive cycle, *etc.*, involved, no general significance is attached to the specific results quoted. The consistency of these results, however, under one set of definitely controlled conditions indicates that the method is valid.

#### SUMMARY

1. A method for measuring the  $O_2$  consumption of a single cell is described. The cell is placed in a capillary tube adjacent to a bubble of air. KOH (5 per cent) is drawn in on the opposite side of the air and both ends of the tube are sealed with mineral oil. The decrease in the volume of the gas, representing the  $O_2$  consumed, is followed.

2. The possible errors of the technique are appraised.

3. A single *Actinosphaerium eichhornii* consumes  $0.00113 \text{ mm.}^3$  of  $O_2$  per hour. A single *Paramecium caudatum* consumes  $0.00049 \text{ mm.}^3$  of  $O_2$  per hour.

4. The significance of the results and the limitations of the method are discussed.

The authors wish to express their thanks to Dr. W. M. Clark and Dr. Barnett Cohen for their kindness in reading and criticizing the manuscript.

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# THE CONDITIONS OF RECOVERY OF TRANSMISSIVITY OF NEWLY REPASSIVATED IRON WIRES IN NITRIC ACID

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In strong nitric acid (*e.g.* 70 volumes per cent<sup>1</sup>) the activation reaction travels over the surface of the passive iron wire in the form of a clearly defined area or "wave" of limited length. In a typical circuit transmission in a pure iron wire (*e.g.*, Armco wire) suspended in  $\text{HNO}_3$  of 78–80 v. per cent at 15° this wave has a length of 12 to 15 cm. and a speed of *ca.* 15 cm. per second.<sup>2</sup> The length ( $L$ ) is determined by the relation between the speed of transmission ( $S$ ) and the duration of the reaction ( $T$ ) at each element of surface (*i.e.*,  $L = ST$ ), and decreases as the concentration of  $\text{HNO}_3$  increases. At any point on the surface the metal reacts with the acid for a brief time (*ca.* 1 second in the instance cited) and instantly becomes passive again. The duration of the local reaction is thus given if we measure the speed and the length of the wave; this is most conveniently done in wires *enclosed in glass tubes containing the acid*,<sup>3</sup> using a millimeter rule and stop-watch. Two processes are thus concerned in the formation of the wave; the first is the local activating process of electrochemical reduction, which disrupts the passivating film and allows metal and acid to interact; the second is the oxidative reaction which restores the film and repassivates the wire. Transmission depends on the first; the second may be compared with the recovery process in the irritable living element; *i.e.*, it is similar in its general relations to the process occurring during the earlier part of the refractory period, by which the just activated nerve fibre or muscle cell regains its tempo-

<sup>1</sup> 70 volumes of  $\text{HNO}_3$ , C.P., sp. gr. 1.42, in 100 volumes of solution.

<sup>2</sup> Lillie, R. S., *Science*, 1929, 69, 305.

<sup>3</sup> Lillie, R. S., *J. Gen. Physiol.*, 1924–25, 7, 477.

rarily lost reactivity. In strong acid ( $> 55$  v. per cent) the wire always shows this prompt spontaneous repassivation.

Whatever may be the conditions in nerve or muscle, we know that in the iron wire the recovery process consists in the production of a new surface film having the same properties as the original film before activation. Repassivation, however, constitutes only one part of the whole recovery process. In general, two distinct phases may be distinguished in this process: first, the formation of the adherent and impermeable oxide film which confers passivity: this occurs rapidly, its most definite index being the electromotor variation of passivation;<sup>4</sup> second, the more prolonged phase during which the repassivated wire, which at first transmits imperfectly, regains by degrees its original transmissivity. Schematically we may represent the sequence of events thus:

*Recovery Process*

- (1) Phase of repassivation
- (2) Phase of imperfect transmission
  - (a) period of decremental transmission
  - (b) period of complete but relatively slow transmission

When we activate a series of similar passive wires in 70 v. per cent  $\text{HNO}_3$  at regularly increasing intervals after a previous activation the phenomena of transmission are observed to vary with time in the following manner. At first the transmission is typically decremental; *i.e.*, the activation wave travels for only a limited distance and then stops short. As time advances the distance travelled increases, and eventually transmission becomes again complete, although at first it is slower than in a completely recovered wire; by degrees it regains its original speed.<sup>5</sup> A similar variation in transmissive properties has been shown to occur in muscle and nerve during the refractory period.<sup>6</sup>

<sup>4</sup> Cf. the string galvanometer curves in Fig. 8 of my recent paper in *J. Gen. Physiol.*, 1929-30, 13, 1.

<sup>5</sup> Lillie, R. S., *J. Gen. Physiol.*, 1920-21, 3, 107.

<sup>6</sup> *I.e.*, the speed of transmission increases progressively during the relative refractory period. For muscle (cardiac) cf. Mines, G. R., *J. Physiol.*, 1913, 46, 349; Ashman R., *Am. J. Physiol.*, 1925, 74, 121; for nerve cf. Forbes, A., Ray, L. H., and Griffith, F. R., Jr., *Am. J. Physiol.*, 1923, 66, 553; Gasser, H. S., and

The duration of the entire recovery period varies with the nature of the wire, the concentration of  $\text{HNO}_3$ , the presence of foreign (e.g. surface-active<sup>7</sup>) substances, and the temperature.<sup>8</sup> In pure soft iron wire in 70 v. per cent  $\text{HNO}_3$  at 20° it lasts for only a few seconds,<sup>9</sup> while in steel (piano) wire it lasts 15 minutes or more.<sup>3</sup> The precise reason for this difference of behavior in different kinds of wire is unknown, but is apparently related to the special structure of the metallic surface.

The present observations were made with steel wires and have reference to the minimal time required for the wire, activated in strong acid and immediately immersed in a known solution of  $\text{HNO}_3$ , to recover its ability to transmit completely or non-decrementally *in strong acid*. In these experiments recovery (apart from the automatic repassivation) thus proceeds in a different concentration of acid (in most cases) from that in which the wire was originally activated. What is determined is the relation between the concentration of the acid surrounding the wire during the second part of the recovery period and the rate at which it reaches a certain definite stage of recovery, as shown by its ability to transmit completely when transferred to strong acid and then immediately activated.

It should be noted that in each experiment the wire is exposed successively to three separate baths of nitric acid: these are (1) the bath (usually of 70 v. per cent  $\text{HNO}_3$ ) in which it is first activated (called Bath A); (2) the bath of varying concentration to which it is

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Erlanger, J., *Am. J. Physiol.*, 1925, 73, 613. In compressed cardiac muscle the early stage of block (which later gives way to complete but slow transmission) corresponds to the decremental stage. It is uncertain at present whether a decremental stage exists during the so-called absolute refractory period of nerve.

<sup>7</sup> The period of repassivation is prolonged several times in 70 v. per cent  $\text{HNO}_3$  to which a small quantity of a surface-action compound, e.g., 0.5 per cent amyl alcohol (soon oxidized to caproic acid), is added. This is an effect of interference with the surface reaction. Correspondingly transmission is greatly slowed in such a mixture.

<sup>8</sup> For the temperature influence cf. Lillie, R. S., *J. Gen. Physiol.*, 1924-25, 7, 477.

<sup>9</sup> Complete transmission returns within a second or less in Armco wire under these conditions,<sup>2</sup> but several seconds more are required before the original speed is regained.

then immediately transferred and in which it lies undisturbed for the measured time allowed for recovery (Bath B); and (3) the bath containing strong  $\text{HNO}_3$  (70 v. per cent or 100 v. per cent in these experiments) to which it is transferred after the lapse of the period of recovery; in this bath (Bath C) it is immediately activated by touching at one end with zinc; the distance travelled by the activation wave is then measured. The transfers are made with platinum-tipped steel forceps.

The wire used was steel wire<sup>10</sup> of the same kind as in my former experiments on transmission and recovery.<sup>3</sup> The lengths of wire, 50 cm. each, were partly dissolved in nitric acid to remove the surface layer of metal, a procedure favorable to uniformity of behavior.<sup>3</sup> In each experiment several completely recovered passive wires were first placed side by side in a porcelain trough containing 70 v. per cent  $\text{HNO}_3$  (Bath A). They were then activated simultaneously (within 2 or 3 seconds) and immediately transferred to a second trough (Bath B) containing the acid whose influence on recovery was to be tested. After definite periods of time in this acid the wires were transferred singly to the strong acid (70 v. per cent or pure 1.42  $\text{HNO}_3$ ) in which the test for transmissivity was made (Bath C) and immediately activated. From previous experiments the probable limits of the recovery times were already known, and the exposures of the several wires in each experiment were timed so as to include a series of partial recoveries (shown by decremental transmission) in addition to the complete recovery. The intervals between the successive transfers varied (according to the strength of acid used in the different experiments) between  $\frac{1}{4}$  minute and 2 minutes. This interval in any single determination represents the approximation to the true recovery time.

Protocols of a number of experiments are given in Table I to illustrate the kind and degree of the variations observed in different solutions of acid.

It will be noted that complete recovery during immersion in 30 v. per cent  $\text{HNO}_3$  required 22 minutes in one case, 23 minutes in another; in 50 v. per cent  $\text{HNO}_3$  the wires in all three experiments were com-

<sup>10</sup> No. 20 piano wire (music steel wire) from the Spencer Co., Worcester, Massachusetts.

pletely recovered after 17 minutes, 16 minutes being nearly sufficient in one case; in 80 v. per cent  $\text{HNO}_3$ , an exposure of  $11\frac{1}{2}$  minutes was sufficient in two cases and nearly so in the third.

Before considering the general results of these measurements, certain peculiarities in the behavior of wires activated while immersed in weak acid should be described. The description of the recovery process on page 350 has had reference to wires activated in strong acid and allowed to recover in the same acid. If passive wires are activated in weak acid (of 50 v. per cent or less) the phenomena are quite different. A wire activated in strong acid, immediately transferred to weak acid, and there reactivated by touching with zinc, instantly

TABLE I

Distances travelled<sup>11</sup> by activation waves in wires activated in pure 1.42  $\text{HNO}_3$ , after having been exposed to  $\text{HNO}_3$  of varying concentrations at 20° for varying periods of time after a previous activation in 70 v. per cent  $\text{HNO}_3$ .

| A. 30 v. per cent $\text{HNO}_3$ |                 |        | B. 50 v. per cent $\text{HNO}_3$ |                 |        |        | C. 80 v. per cent $\text{HNO}_3$ |                 |        |        |
|----------------------------------|-----------------|--------|----------------------------------|-----------------|--------|--------|----------------------------------|-----------------|--------|--------|
| Exposure                         | Distances (cm.) |        | Exposure                         | Distances (cm.) |        |        | Exposure                         | Distances (cm.) |        |        |
|                                  | Exp. 1          | Exp. 2 |                                  | Exp. 1          | Exp. 2 | Exp. 3 |                                  | Exp. 1          | Exp. 2 | Exp. 3 |
| <i>minutes</i>                   |                 |        | <i>minutes</i>                   |                 |        |        | <i>minutes</i>                   |                 |        |        |
| 16                               | 8.3             | 10.0   | 12                               | 10.1            | 9.1    | 8.4    | 9                                | 14.8            | 13.3   | 13.0   |
| 20                               | 27.5            | 21.8   | 14                               | 11.2            | 13.2   | 18.4   | 10                               | 23.2            | 21.1   | 15.8   |
| 22                               | 34.2            | Full   | 16                               | 22.7            | 26.3   | 42.0   | 11                               | 28.1            | 30.3   | 18.0   |
| 23                               | Full            |        | 17                               | Full            | Full   | Full   | $11\frac{1}{2}$                  | Full            | Full   | 38.3   |

transmits rapidly along its whole length and *remains* active, *i.e.*, effervescence and solution continue unchecked until the whole wire is dissolved. In this case the decremental stage is entirely absent,

<sup>11</sup> In my earlier paper of 1920,<sup>8</sup> transmission for a distance of 20 cm. was regarded as evidence of full recovery. I have since found, by the use of longer troughs, that transmission waves, especially in stronger acid (*e.g.* 1.42  $\text{HNO}_3$ ) will at times (although infrequently) come to a stop after travelling for 30 or 35 or even (in a few cases) 40 cm. along the wire. Transmission for 50 cm. may safely be regarded as complete. It should be remembered, however, that accidental irregularities in the composition of the metallic surface may at times interfere with transmission in wires otherwise completely recovered.

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Before considering the general results of these measurements, certain peculiarities in the behavior of wires activated while immersed in weak acid should be described. The description of the recovery process on page 350 has had reference to wires activated in strong acid and allowed to recover in the same acid. If passive wires are activated in weak acid (of 50 v. per cent or less) the phenomena are quite different. A wire activated in strong acid, immediately transferred to weak acid, and there reactivated by touching with zinc, instantly

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|----------------------------------|-----------------|--------|----------------------------------|-----------------|--------|--------|----------------------------------|-----------------|--------|--------|
| Exposure                         | Distances (cm.) |        | Exposure                         | Distances (cm.) |        |        | Exposure                         | Distances (cm.) |        |        |
|                                  | Exp. 1          | Exp. 2 |                                  | Exp. 1          | Exp. 2 | Exp. 3 |                                  | Exp. 1          | Exp. 2 | Exp. 3 |
| <i>minutes</i>                   |                 |        | <i>minutes</i>                   |                 |        |        | <i>minutes</i>                   |                 |        |        |
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transmits rapidly along its whole length and *remains* active, *i.e.*, effervescence and solution continue unchecked until the whole wire is dissolved. In this case the decremental stage is entirely absent,

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as is also the spontaneous repassivation or recovery.<sup>12</sup> Such an experiment is instructive in several respects; it shows (what was already known<sup>13</sup>) that automatic repassivation requires a certain minimal concentration of  $\text{HNO}_3$ ; it also shows that the condition which determines repassivation is the same as the condition which renders the wire for a time decrementally instead of completely transmissive. This condition may be defined as more than a certain critical intensity of oxidative action.

It is evident that decremental transmission depends on some special peculiarities of the newly formed passivating film, since it disappears if the passive wire remains for a certain time in the acid. The nature of the conditions may perhaps best be indicated as follows. If we take a wire at a certain stage of recovery in 70 per cent  $\text{HNO}_3$ , *e.g.* one minute after activation in this solution at  $20^\circ$ , we find that it transmits completely if transferred to 50 per cent  $\text{HNO}_3$  and then activated; but only for 1 or 2 cm. if activated in 70 per cent acid, and hardly at all in 100 per cent (1.42) acid. In general, at a given interval after a previous activation the distance of transmission is less (*i.e.*, the degree of decrement is greater) the stronger the acid in which the wire is activated.<sup>13</sup> At the region occupied by the activation wave each element of surface is the seat of two distinct chemical processes which are opposed in their general effect, the one being reducing (film-disruptive), the other oxidative (film-forming). The relative intensities of these two processes determine which one gains the upper hand. If the oxidative process has more than a certain intensity, the surface film of oxide rapidly becomes continuous and impermeable to acid, and the reaction ceases; if it has less than this intensity, no effective or stable film is formed and the reaction continues unchecked.

<sup>12</sup> This does not mean that a newly repassivated wire left undisturbed in weak acid shows no phenomena of recovery; such a wire, in fact, shows the same kind of change as a wire in strong acid during the period succeeding the decremental phase; *i.e.*, the speed with which it transmits is relatively slow at first and becomes more and more rapid as time elapses up to a maximum. This can most readily be shown by timing with a stop-watch the speeds of transmission of a series of passive wires in glass tubes containing the weak acid, at varying intervals after the previous activation in strong acid.

<sup>13</sup> Cf. my early paper on recovery in steel wires, *J. Gen. Physiol.*, 1920-21, 3, 107.

In the case of the typical automatically reversed activation reaction in strong acid it is clear that the surface oxidation which reforms the film also imparts to it at the same time some structural or other character which at first is unfavorable to transmission, as shown by the decremental phase of early recovery. The succeeding change from decremental to non-decremental transmission is the index of some progressive chemical or structural change occurring in the passivating film under the influence of the surrounding acid. Experiments with different concentrations of  $\text{HNO}_3$  show that the rate of this change is a direct function of the concentration. To illustrate: in a series of preliminary observations the wires were activated in 70 v. per cent  $\text{HNO}_3$  and were allowed to recover in acid of the four concentrations given in Table II. The exposures required to render them again completely transmissive in 70 v. per cent  $\text{HNO}_3$  were as follows:

TABLE II

| Concentration<br>(v. per cent $\text{HNO}_3$ of 1.42 sp. g.) | Minimal exposures for restoration of<br>non-decremental transmission in 70 v. per cent<br>$\text{HNO}_3$ (minutes at 19°-20°) |
|--|---|
| 10   | ca. 25  |
| 25   | > 11 (est. ca. 14)  |
| 70   | 6-7   |
| 100  | 3½-4  |

The rate of recovery is thus about seven times as rapid in the concentrated as in the 10 v. per cent  $\text{HNO}_3$ .

In a more complete series of experiments the exposures required to render the newly activated wires completely transmissive in 100 v. per cent (sp. gr. 1.42)  $\text{HNO}_3$  were determined for a series of concentrations between 15 v. per cent and 100 v. per cent. In general these exposures (for any given concentration) are about twice as long as those required to render the wires transmissive in 70 per cent  $\text{HNO}_3$ . This shows again that at a certain stage of recovery the passivating film has properties permitting complete transmission in the weaker but not in the stronger acid. The decremental transmission observed in the stronger acid at a time when the wire transmits freely in 70 per cent acid, is the expression of its more intense oxidative action; this interferes with the local reduction on which transmission depends.

The results of this series are summarized in Table III which gives the average minimal exposures required to render the wires non-decrementally transmissive in 1.42 HNO<sub>3</sub> at 19–20°. The number of separate experiments under uniform conditions (of the kind illustrated in Table I) made with each concentration of HNO<sub>3</sub> in Bath B is stated in brackets in the first column; the second column gives the averages and probable errors of these averages;<sup>14</sup> in the third column are the products of the concentrations into the effective times of exposure.

The results of the two series are represented graphically, with both linear and logarithmic coordinates, in Fig. 1. The former curves are

TABLE III

| Concentration (C)<br>(v. per cent HNO <sub>3</sub> of sp. gr. 1.42)<br>(number of separate determinations in brackets) | Minimal exposures (T) for<br>restoration of non-decremental<br>transmission in 100 v. per<br>cent HNO <sub>3</sub> (minutes at 19°–20°) | CT             |
|--|---|----------------|
| 15 (4)   | <i>ca.</i> 40   | <i>ca.</i> 600 |
| 20 (36)  | 35.5 ± 0.47   | 710            |
| 30 (18)  | 23.4 ± 0.26   | 705            |
| 40 (7)   | 19.6 ± 0.17   | 784            |
| 50 (12)  | 16.6 ± 0.10   | 830            |
| 60 (14)  | 14.1 ± 0.11   | 846            |
| 70 (23)  | 12.4 ± 0.15   | 868            |
| 80 (8)   | 11.4 ± 0.11   | 912            |
| 100 (16)   | 10.5 ± 0.13   | 1050           |

hyperbola-like in their general character, although departing considerably from the rectangularity which would correspond to a direct proportionality between concentration and rate of recovery. This proportionality is approached in the lower concentrations; but above 50 v. per cent the recovery becomes progressively slower, in relation to concentration, as the latter increases. In the curves with logarithmic coordinates the points of both series fall approximately on straight lines, indicating that the film is altered during recovery at a rate closely proportional to the external concentration of acid.

Apparently the passivating film, at its first deposition in the strong

<sup>14</sup> Calculated by Peters' approximation formula (*cf.* Mellor, J. W., Higher mathematics, Longmans Green and Company, London, 1926, 524).

acid, has some structural or other character, such as thickness, irregular orientation of molecules, or possibly a less intimate contact with the metallic surface, which makes it relatively resistant to electrolytic reduction. Whatever the precise conditions may be, it is clear that under the action of the external acid this character changes in such a manner as to render the film progressively more reducible. The final

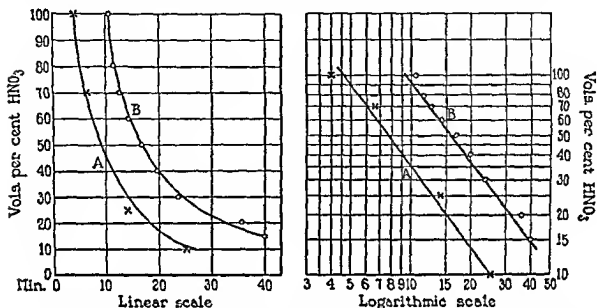


FIG. 1. Times of immersion in HNO<sub>3</sub> of different concentrations required<sup>7</sup> for recovery of complete or non-decremental transmissivity. A, for transmission in 70 v. per cent HNO<sub>3</sub>; B, for transmission in pure (100 v. per cent) HNO<sub>3</sub> (of s.g. 1.42). Temperature 19–20°. Ordinates, vols. per cent HNO<sub>3</sub>; abscissae, minutes. Data are plotted with both Cartesian and logarithmic coordinates.

or limiting state, corresponding to maximal speed of transmission<sup>7</sup> (or stage of complete recovery), is the stage at which the film is most readily reduced. This is shown further by the fact that the threshold of electrical activation of the wires is then lowest.<sup>15</sup>

<sup>15</sup> This can best be shown by determining with the Lucas pendulum the duration of the constant current, of known E.M.F., required to activate the passive wire electrically. I have made numerous experiments of this kind with pure iron wires (Armco) during the past few years; in general under similar conditions of recovery in 70 v. per cent HNO<sub>3</sub> the product  $i\sqrt{t}$  ( $i$  being the intensity and  $t$  the minimal or threshold duration of the activating current) remains nearly constant throughout a wide range of intensities (between 1 and 8 volts). This product is highest shortly after repassivation and decreases progressively as time elapses to a minimum corresponding to complete recovery. A correlation between electrical

The hypothesis of a solvent action by which the outer layers of the oxide sheet (assumed at its first deposition to be several molecules thick) are dissolved away as ferric nitrate seems the best adapted to explain the general course of the recovery process. The same final stage is reached in the different solutions of  $\text{HNO}_3$ , but at a rate which varies with concentration in the manner described. The relation between concentration and rate of recovery is not far from proportional in the weaker solutions up to 50 v. per cent,—*i.e.*, there is an approach to a mass action relationship; but in the stronger solutions the rate of recovery becomes somewhat slower, relatively to concentration, as the latter increases. This may indicate the superposition of two actions, one the direct solvent action of the acid on the film, the other some further oxidative action (possibly intermittent) upon the metal, having the effect of renewing or reinforcing the film wherever it becomes defective and so retarding its dissolution. This apparent interference is well marked in the passivating concentrations ( $> 55$  v. per cent) and becomes more distinct as the concentration increases. Occasionally, under conditions difficult to define, I have found wires to remain decrementally transmissive for abnormally long periods, even several hours, while immersed in pure 1.42  $\text{HNO}_3$ . This refractory behavior is certainly an effect of strong oxidation. On the general view just indicated the state of decremental or slow transmissivity would correspond to the presence of a relatively thick oxide sheet; while maximal speed would indicate minimal thickness, possibly monomolecular. Further thinning in the strong acid would be automatically prevented, because any accidental local interruptions exposing the metal, being local anodes with high current density, would instantly be repaired.<sup>16</sup>

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sensitivity and speed of transmission is thus present in the passive iron system as well as in the irritable living system such as nerve. (For this correlation in nerve *cf.* Erlanger, J., and Gasser, H. S., *Am. J. Physiol.*, 1924, 70, 624; 1930, 92, 43.)

<sup>16</sup> This is a feature of the behavior in strong acid (for a brief discussion of the general conditions *cf.* my paper in *J. Gen. Physiol.*, 1929-30, 13, 1, footnote p. 7). In weak acid such automatic tendency to repair is slight or absent. In fact one practical difficulty in determining the duration of the recovery periods in long passive wires in weak acid is that such wires are likely to become suddenly active, at irregular and unpredictable intervals, without any apparent inciting cause;

With regard to the bearing of these observations on the problem of the conditions of recovery in irritable living tissues such as nerve, only a few general remarks need be made. According to the membrane conception of the primary stimulation process, the fundamental similarity between the iron wire model and the living tissue is that in both systems the material whose chemical reaction furnishes the condition of transmission forms part of a thin, highly impermeable film which is situated at the boundary between two chemically dissimilar phases. The impermeability (or semi-permeability) depends on the special chemical and structural composition of the film; it also furnishes the condition for the characteristic electrical P.D. between the phases. Hence any sufficient local alteration in the film causes a variation of potential and gives rise to local currents between the altered and the unaltered regions. It is because of this special situation of the reactive material in the electrically polarized film that the latter is subject to chemical and structural change under the influence of electric currents passing between the phases; transmission depends directly upon this condition, local circuits, with associated distance-action effects, arising wherever the film is broken down or sufficiently altered.

Accordingly we regard the primary phase of recovery in the irritable living system as consisting in the replacement of some material

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especially in acid of 10 to 20 v. per cent is this spontaneous activation so frequent that many experiments have failed from this cause alone. Evidently the indefinite preservation of passivity depends on local automatic repassivation, *i.e.*, is an active rather than a merely passive phenomenon, resembling in this respect the preservation of semi-permeability in the membranes of living cells. It is interesting to note that Bishop (Bishop, G. H., *J. Gen. Physiol.*, 1927-28, 11, 159) observed an irregular oscillating or flickering effect, the indication of minute local currents, when the passive wire was connected to the cathode ray oscillograph through an amplifier. The instability of the passive state in weak acid is thus readily understood. Correspondingly the threshold of electrical activation is low in such solutions, and in general decreases rapidly with the concentration of acid. In highly dilute acid passive wires are activated with extreme ease, mechanically or otherwise. It would seem that in wires lying undisturbed in such acid any minute local area where the film becomes discontinuous is not repaired but instantly spreads. The time and locus of such an occurrence are apparently a matter of chance, hence short wires will (as a rule) remain passive for much longer periods than long wires.

which is chemically broken down in the reaction of activation; this material is essential to the normal semi-permeability and electrical polarization of the plasma membrane. But in addition to this replacement some further change, partly chemical, partly structural, seems to be required in both the living and the non-living systems. This change occupies the relative refractory period; its nature in the iron wire is partly indicated by the foregoing experiments, but with regard to the living system only conjecture is possible at present. There is much evidence that the characteristic semi-permeability of the living plasma membranes is dependent on their lipoid components; but we have as yet no clear evidence that lipoids are altered during transmission, although in the case of nerve the production of ammonia<sup>17</sup> and the negative fact of an absence of increase in carbohydrate metabolism<sup>18</sup> may seem to point in this direction. Comparative metabolic studies on a variety of irritable tissues offer the best prospect of throwing light on this problem. The key to the situation is the constitution of the plasma membrane of the irritable element. Provisionally we may conceive of this membrane as a mosaic-like structure in which the channels permitting diffusion of ions are blocked during rest by the reactive material in question—possibly lipoid. This material, broken down during stimulation, is restored and rearranged during the recovery period.

#### SUMMARY

1. Passive steel wires were activated in a bath (Bath A) containing 70 v. per cent  $\text{HNO}_3$  (in which they undergo prompt repassivation), and immediately transferred to a second bath (Bath B) containing  $\text{HNO}_3$  of a concentration varying in different experiments. After varying intervals in this bath they were transferred while still passive

<sup>17</sup> Tashiro, S., *Am. J. Physiol.*, 1922, 60, 519; Gerard, R. W., and Meyerhof, O., *Biochem. Z.*, 1927, 191, 142.

<sup>18</sup> Gerard and Meyerhof found no change in lactic acid production during the stimulation of nerve (Gerard, R. W., and Meyerhof, O., *Biochem. Z.*, 1927, 191, 125). In a recent paper (*Am. J. Physiol.*, 1930, 93, 342) Holmes, Gerard, and Solomon have described experiments showing that stimulated nerve consumes no more carbohydrate than resting nerve. There is also evidence of increase in the soluble phosphate of nerve during activity, a fact again favoring the view that lipoids are concerned in transmission (*cf.* Gerard, R. W., and Wallen, J., *Am. J. Physiol.*, 1929, 89, 108).

to a third bath (Bath C) containing strong  $\text{HNO}_3$  (70 or 100 v. per cent) and there immediately activated.

2. During the immersion in Bath B the wires progressively recover their ability to transmit activation waves in strong  $\text{HNO}_3$ . The measure of this recovery is the distance travelled by the activation waves in Bath C after the varying times of exposure in Bath B. Transmissivity as thus measured is at first incomplete (decremental) and later becomes complete. The minimal exposures in Bath B required to render wires completely transmissive in the strong acid of Bath C were determined for concentrations of  $\text{HNO}_3$  between 10 and 100 v. per cent. With 100 v. per cent  $\text{HNO}_3$  in Bath C, these exposures range from 40 minutes or more in 15 v. per cent to 10 minutes in 100 v. per cent  $\text{HNO}_3$  (temperature 19–20° in all baths).

3. The time required for complete recovery varies inversely with the concentration of the acid in the recovery bath (Bath B), but increases rapidly with the concentration of the acid in the testing bath (Bath C). Hence at a time when a wire has recovered just sufficiently to transmit non-decrementally in a given strong acid (e.g., 70 v. per cent) it still transmits decrementally in a stronger acid. Complete recovery for transmission in 100 v. per cent  $\text{HNO}_3$  requires about twice as long as for 70 v. per cent  $\text{HNO}_3$ . In  $\text{HNO}_3$  of 50 v. per cent and less decremental transmission does not occur.

4. The indications are that recovery is an effect of the progressive solvent action of the external acid on the passivating oxide film, which at its first deposition appears to be relatively thick and hence resistant to electrochemical reduction. The final stage of recovery, when electrical sensitivity and speed of transmission are maximal, would on this hypothesis correspond to minimal thickness, possibly monomolecular.

5. The rate of recovery in Bath B is not far from proportional to the concentration of  $\text{HNO}_3$  in the more dilute solutions, but in the higher, especially the strongly passivating, concentrations (70 to 100 v. per cent) the rate becomes appreciably slower than proportional, apparently because of the intense oxidizing action of these solutions, which reinforces the oxide sheet and retards the thinning process.

6. The bearing of these observations on the problem of the conditions of recovery in irritable living tissues (such as nerve) during the absolute and relative refractory periods is briefly discussed.





## THE CAMBIUM AND ITS DERIVATIVE TISSUES\*

### VI. THE EFFECTS OF HYDROGEN ION CONCENTRATION IN VITAL STAINING

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#### INTRODUCTION

The cambium and its derivatives provide an unusually favorable medium for the study of living tissue cells of the higher plants. The uninjured cells in the interior of skillfully prepared sections have been kept alive and with the protoplasm *actively streaming* for 1710 hours in tap water, 1230 hours in distilled water, 500 hours in medicinal liquid petrolatum, from 300–1100 hours in various salt solutions, and from 120–500 hours in solutions of sucrose varying in concentration from  $\frac{M}{8}$  to  $\frac{10M}{8}$ . In other words except at the height of the growing season, the tissues are extraordinarily resistant to mechanical injuries<sup>1</sup> and to drastic changes of environmental factors,—e.g., temperature, light, pH, osmotic pressure, etc. Because of the size, form, and orientation of the cells, such structures as the nucleus, nucleoli, vacuoles, plastids, and phragmoplasts, and not infrequently the mitochondria, are clearly visible in the living, unstained protoplasts. Furthermore, the *visible* structural changes induced in the cells by traumatic and other abnormal stimuli occur relatively slowly. Since sections of the tissues can be cut and placed under microscopic observation in less than 60 seconds, it is possible to study the tissue cells before they have

\* This investigation was supported in part by special grants from the Carnegie Institution of Washington and the Milton Research Fund of Harvard University.

<sup>1</sup> The tissues normally are subjected to violent stresses and strains during the swaying of tall trees in the wind.

had time to become visibly modified and to observe directly the successive structural changes that are induced by specific combinations of environmental factors.<sup>2</sup>

In the investigations described in the preceding paper (1) of this series, two types of vacuoles were encountered in the living cells of the cambium and of its derivative tissues. The vacuoles of one of these categories, *A-type*, become intensely magenta or red in aqueous solutions of either neutral red or methyl red; those of the other category, *B-type*, stain orange or reddish-orange in aqueous solutions of neutral red, but give no color reactions with methyl red. Vacuoles of the former type accumulate dyes rapidly from both relatively alkaline (neutral red) and relatively acid (methyl red) solutions; whereas vacuoles of the latter type are able to do so from relatively alkaline (neutral red) solutions only. In view of the fact that both types of vacuoles frequently occur in more or less close contact *within the same protoplast*, it is evident that these variations in staining reactions must be due to fundamental differences in the vacuoles themselves rather than to putative differences in the cell walls, protoplasmic membrane, or cytoplasm. In other words, absence of vital staining of the B-type vacuoles in methyl red is not due to a failure of the dye to penetrate cells which contain these vacuoles.

#### *Distribution of A-Type and B-Type Vacuoles in the Higher Plants*

That the A-type and B-type vacuoles are not of isolated or sporadic occurrence, but are to be found throughout the higher plants has been determined by Miss Anna F. Faull of this laboratory in a special survey of 272 representatives of 42 orders and 90 families of the Pteridophyta, Gymnospermae, and Angiospermae. Although the abundance and distribution of the two categories of vacuoles vary markedly from plant to plant, from organ to organ, and from tissue to tissue, none of the species studied is entirely devoid of either the A-type or the B-type vacuoles.

The A-type vacuoles, which stain vitally with both neutral red and methyl red, contain certain categories of aromatic substances,—*i.e.*,

<sup>2</sup> The tropistic responses of the massive stems of trees are, in general, relatively sluggish. There are no "trigger" mechanisms or special adaptations for the rapid transmission of stimuli.

phenolic compounds, tannins, flavones, flavonols, anthocyanins, and tend to form copious deeply-colored precipitates. The typical B-type vacuoles, which do not stain vitally with methyl red, do not contain microscopically detectable quantities of these aromatic compounds and rarely form massive intravacuolar precipitates. Where precipitation occurs in connection with the vital staining of B-type vacuoles in neutral red, the relatively scanty, colored precipitate commonly appears on, and adheres to, the outer surface of the vacuoles. In certain cases, one or more colored granules, crystals, or spherocrystals may be formed internally. In differentiating tissues, A-type vacuoles may metamorphose at times from B-type vacuoles. Thus, transitional or intermediate types of vacuoles are of not infrequent occurrence.

It is evident, therefore, that there are at least two distinct categories of vacuoles in the higher plants which should be considered in any general discussion of vital staining. Many of the serious discrepancies in the literature relating to the penetration and accumulation of dyes in living plant cells may be due to the fact that different investigators are concerned with different categories of vacuoles. The physico-chemical mechanism of the accumulation of dyes in the vacuoles of *Valonia* and *Nitella*, which are apparently of the general B-type, may be quite different from that in the case of A-type vacuoles.

In view of these facts, it seemed advisable to the writers to initiate an intensive study of the phenomena of vital staining in the cambium and its derivative tissues, particularly of the effects of hydrogen ion concentration upon the accumulation of various dyes in A-type and B-type vacuoles.

### *Criteria for Distinguishing Living from Dying and Dead Cells*

Much confusion has been introduced into biological literature by applying the term "vital" to those types of staining which immediately precede, or are concomitants of, the degeneration and death of the cell. It is indispensable, in dealing with truly vital staining, to secure reliable criteria for distinguishing living from dying and dead cells and to keep an accurate record of the length of time that cells survive after a given observation or experiment is completed.

Various criteria of "vitality" that have been used in cytological and physiological investigations have been tested by the writers and found to be unreliable. The most serviceable and dependable criterion in the case of the cambium and its

derivatives is the phenomenon of protoplasmic streaming,—i.e., *cyclosis*. Streaming cells may safely be regarded as living. In addition, the phenomena of normal cyclosis,<sup>3</sup> particularly in the Gymnosperms, are so characteristic and so easily modified that they provide extremely sensitive indicators for detecting abnormalities, incipient injuries, and irreversible changes in the cytoplasm.

In certain of the fully differentiated derivatives of the cambium, the cytoplasm normally is not in active cyclosis, at least during certain periods of the year. When streaming cannot be induced in such cells, the writers have been forced to rely upon cumulative evidence in distinguishing the living from the seriously injured and dead cells. No observations are recorded from cells which exhibit any of the following symptoms: (1) Conspicuous opacity; (2) granulation or coagulation of the cytoplasm or of the contents of the nucleus; (3) active Brownian movement of ergastic particles within the cytoplasm; (4) aggregation or agglutination of ergastic particles within the cytoplasm; (5) visible staining of the nucleus, nucleoli, mitochondria, or cytoplasm. Cells which exhibit none of these symptoms are regarded as living if they are capable of plasmolyzing and of deplasmolyzing in sugar solutions.<sup>4</sup>

The only available means, at present, for detecting *invisible*, irreversible injuries—which may lead to a gradual, rather than to a sudden, death of the cell—is the study of the record of survival.<sup>5</sup> In other words, cells which survive for a specified interval are not considered to have been injured irreversibly at the beginning of that interval. The length of this test-period must be determined more or less arbitrarily and must be varied to meet the requirements of different organisms, tissues, and experimental procedures.

### Controls

That living cells can be removed from the tree without seriously injuring them is shown by the fact that “explants” of *Pinus Strobus* L. have been kept alive and actively streaming for more than two months (1710 hours) in running tap water. The only conspicuous change in the cytoplasm during the interval is a gradual

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<sup>3</sup> The problem of distinguishing normal from traumatically induced cyclosis was discussed in the preceding paper of this series, Bailey (1).

<sup>4</sup> Under certain conditions dead protoplasts may contract and expand, thus simulating plasmolysis and deplasmolysis. Although the two categories of phenomena may readily be distinguished by an experienced investigator, they appear to have been confused, at times, by incautious observers.

<sup>5</sup> Reversible and irreversible changes have been detected by bioelectrical methods, but there are serious difficulties to be overcome in so studying cells in tissues. Even in the case of *Valonia* and *Nitella*, such techniques have not been utilized in experiments dealing with the penetration of dyes. The condition of specific cells during experimentation is judged by observing the behavior of “controls” transferred to normal environmental conditions.

disappearance of ergastic particles,—e.g., oil globules. This suggests that in tap water, distilled water, and mineral oils the uninjured cells function as more or less independent and self-sustaining units, and that they are capable of maintaining cyclosis until the internal supply of available energy is exhausted.<sup>6</sup>

Uninjured cells survive for from 700 to 1500 hours when kept in stagnant tap water. Furthermore, the cells commonly remain alive and actively streaming for several hundred hours even when the sections become so completely encrusted with bacteria, yeasts, and other microorganisms that they must be scraped before they can be examined microscopically. Sections in tap water may, therefore, be utilized in experimental work as convenient controls for determining whether the cells have been injured in sectioning.

### *Toxicity of Buffered Solutions*

(A) *Clark's Standard  $\frac{M}{20}$  Buffers.*—In studying the effects of extracellular pH upon the staining of living cells, it is essential to avoid the use of highly toxic buffers. The cambial cells of *Pinus Strobus* L. seldom survive for any considerable time in Clark's (2)  $\frac{M}{20}$  buffers except in the pH 5.4 and pH 5.8 phthalates, the pH 5.8 and pH 6.2 phosphates, and the pH 7.8 and pH 8.2 borates. The death of the cells is due to the buffers rather than to the effects of sectioning or of subsequent manipulation of the sections. This is clearly demonstrated by the fact that duplicate sections placed in tap water remain alive and actively streaming for several hundred hours.

(B)  *$\frac{M}{20}$  Buffers Plus Sucrose.*—The addition of sugar to make the buffers more nearly "isotonic" tends to prolong the life of the cells somewhat, but the general pattern of survival throughout the pH range is similar to that in the case of the standard  $\frac{M}{20}$  buffers. In both sets of buffers there are conspicuous lethal effects in the more alkaline borates and phosphates and in the more acid phthalates.

(C)  *$\frac{M}{40}$  Buffers.*—Dilution of the standard buffers to  $\frac{M}{40}$  also tends to prolong the life of the cells, but, as in the case of the saccharine buffers, without fundamentally changing the general pattern of survival.

In the preparation of the borate and phosphate buffers  $H_2BO_3 + KCl$  and  $KH_2PO_4$  are constants, whereas the quantity of NaOH increases markedly between pH 6.2 and pH 7.8 phosphate and between pH 8.2 and pH 10.0 borate. This raises the question whether the increasing toxicity of the more alkaline phosphate and borate buffers may be due largely to sodium poisoning, and whether the effects of the sodium<sup>7</sup> may be antagonized by relatively minute quantities of calcium.

<sup>6</sup> We are dealing here with cells which do not contain chloroplasts.

<sup>7</sup> In reality sodium plus potassium.

(D)  $\frac{M}{40}$  *Buffers Plus Calcium Chloride*.—That these questions may be answered in the affirmative, at least in so far as the phosphate buffers are concerned, seems probable. When two drops of  $\frac{M}{5}$   $\text{CaCl}_2$  are added to 50 cc. of each of the  $\frac{M}{40}$  buffers, the cells remain alive and actively streaming for more than 120 hours throughout the range pH 5.0–pH 8.6. Experiments with various acids and alkalis suggest that the toxicity of the buffers in the pH 3.0 to pH 4.6 range is due primarily to excessive acidity; that in the pH 9.0 to pH 10.0 range largely to excessive alkalinity.

To attempt to prolong the life of the cells beyond 200 hours by further dilutions of the buffers or by the addition of sugar, larger quantities of calcium, etc., leads to unavoidable complications and possibly to serious errors, and is not justifiable in the type of experimental investigations undertaken by the writers.

### *Toxicity of Dyes of Buffered Solutions*

The toxic effects of each dye—or rather of each dye *plus its impurities*—may be tested by comparing the record of survival of uninjured cells placed in two series of buffers, one with and one without the dye.

Most dyes produce conspicuous lethal effects, at least in certain of the buffers. It is essential, accordingly, to determine how long the cells may remain in each buffered solution of the dye without sustaining serious irreversible injuries. When the dyes accumulate rapidly and the cells survive for many hours, the staining may be regarded as truly vital. On the contrary, when the cells survive for a limited period only, the staining may be a concomitant of, or may be modified by, irreversible changes in dying cells. Valuable clues concerning the magnitude of such injuries at any specific stage of staining may be obtained, however, by transferring the sections to dye-free buffer or to tap water.

### *Miscellaneous Comments upon Materials and Methods*<sup>3</sup>

In dealing with vital staining, it is essential that the sections be of uniform thickness and that such general environmental factors as light and temperature be kept approximately constant. Of course, the usual precautions must be observed in the preparation of buffers,—*i.e.*, use of special reagents and glassware, periodic verifications of pH, etc. Seventeen buffers are used by the writers. The two pH 5.8 buffers facilitate comparisons between phthalates and phosphates, and the two pH 7.8 buffers, between phosphates and borates.

In the case of the B-type vacuoles, three intensities of staining may be distinguished visually; (1) light, (2) medium, and (3) intense. In the case of the

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<sup>3</sup> For a detailed discussion of our technique for studying living tissue cells and for distinguishing vacuoles from other constituents of the cell, the reader is referred to the preceding paper of this series (1).





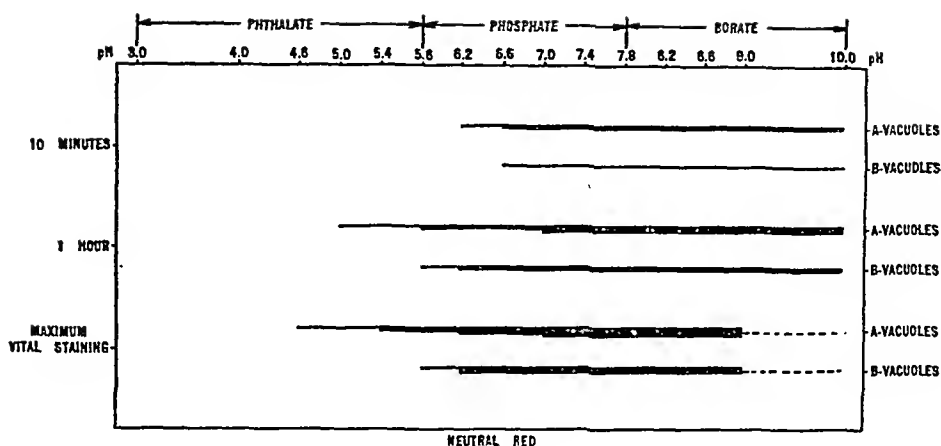


FIG. 1. Ranges and intensities of vital staining of A-type and of B-type vacuoles in buffered solutions of neutral red. Varying intensities are indicated by lines of varying width. - - - data complicated by lethal factors.

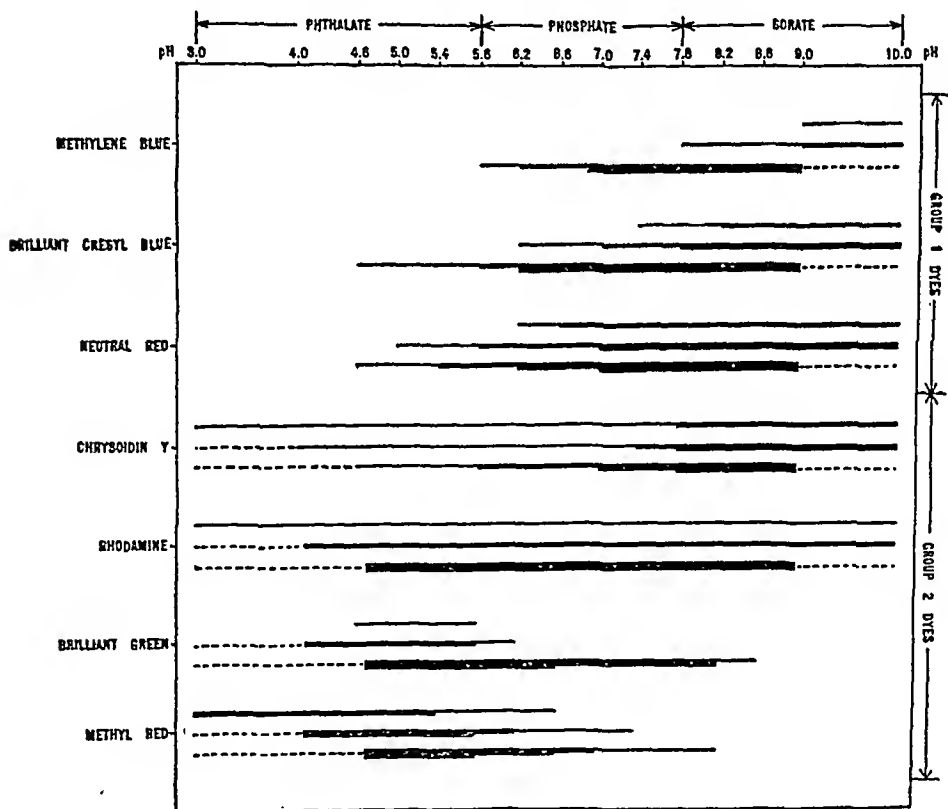


FIG. 2. Ranges and intensities of vital staining of A-type vacuoles in buffered solutions of Group I and Group II dyes. The time intervals in each case are 10 minutes, 1 hour, and period of maximum vital staining. Varying intensities of vital staining are indicated by lines of varying width. - - - data complicated by lethal factors.

vacuoles more rapidly and intensely from alkaline than from acid buffers; slightly or not at all from buffers more acid than pH 5.4–pH 5.8. As indicated in Fig. 1, the A-type vacuoles stain more rapidly and over a wider range of acid buffers than do the B-type vacuoles. The latter rarely, if ever, accumulate the dyes from buffers more acid than pH 5.4–pH 5.8.

The rate of staining increases more or less rapidly with increasing alkalinity until a certain degree of alkalinity is reached, beyond which no marked fluctuations in the rate of staining can be detected by visual means. The dyes accumulate in the vacuoles until certain maximum intensities of staining are attained. The ranges, rates, and intensities of staining vary more or less from dye to dye, from species to species, and from tissue to tissue, and apparently also in buffers of varying composition. Sugar tends to retard and calcium to accelerate vital staining.

## GROUP II DYES

*Dyes Which Accumulate in A-Type But Not in B-Type Vacuoles*

| Dye                             | Maker      | Lot number |
|---------------------------------|------------|------------|
| Basic fuchsin                   | NAC Co.    | NF-4       |
| Benzene-azo-alpha-naphthylamine | C & B Co.* |            |
| Bismarck brown                  | NAC Co.    | 2292       |
| Brilliant green                 | Grübler    |            |
| Celestin blue                   | "          |            |
| Chrysoidin R                    | NAC Co.    | 2246       |
| Chrysoidin Y                    | "          | 4050       |
| Crystal violet                  | C & B Co.  |            |
| Dahlia (Hoffman's violet)       | NAC Co.    | 4316       |
| Ethyl red                       | C & B Co.  |            |
| Gallocyanine                    | "          |            |
| Gallamine blue                  | Grübler    |            |
| Janus green B                   | Hoechst    |            |
| Malachite green                 | NAC Co.    | NMg-2      |
| Methyl red                      | "          | 2338       |
| Methyl violet 2B                | Grübler    |            |
| Neutral blue                    | "          |            |
| Nile blue BB                    | NAC Co.    | 2778       |
| Propyl red                      | C & B Co.  |            |
| Pyronin B                       | NAC Co.    | NP-2       |
| Rhodamine (vital)               | "          | 5140       |
| Safranin bluish                 | "          | 4568       |
| Safranin O                      | "          | 2066       |

\* C and B Co. = Coleman and Bell Co.

As shown in Fig. 2, the behavior of these dyes in the staining of A-type vacuoles is extremely variable. Such dyes as chrysoidin Y resemble the Group I dyes in staining more rapidly and intensely in alkaline than in acid buffers. On the contrary, such dyes as methyl red, ethyl red, and brilliant green accumulate rapidly from acid buffers; slowly, if at all, from alkaline buffers. Rhodamine accumulates at a relatively uniform rate throughout the pH range. It appears to do so even in dilutions of .0005 per cent. Safranin bluish tends to stain more rapidly in acid and alkaline than in more nearly neutral buffers; whereas Nile blue BB does the reverse. Bismarck brown appears to be intermediate in its general staining reactions between chrysoidin Y and rhodamine; benzene-azo-alpha-naphthylamine, between methyl red and rhodamine, etc. As in the case of the Group I dyes, the details of vital staining vary somewhat from species to species and from tissue to tissue.

## GROUP III DYES

*Dyes Which Do Not Accumulate in Either A-Type or B-Type Vacuoles*

| Dye                        | Maker       | Lot number |
|----------------------------|-------------|------------|
| Acid fuchsin.....          | PC Co.*     |            |
| Alizarin red S.....        | NAC Co.     | 3825       |
| Amaranth.....              | "           | 1028       |
| Aniline blue.....          | "           | 1137       |
| Aniline yellow.....        | "           | 3791       |
| Benzopurpurin 4B.....      | "           | 1083       |
| Biebrich scarlet.....      | "           | 2245       |
| Bordeaux red.....          | "           | 2130       |
| Brom cresol green.....     | La Motte    |            |
| Brom cresol purple.....    | "           |            |
| Brom phenol blue.....      | "           |            |
| Brom thymol blue.....      | "           |            |
| Chlor phenol red.....      | "           |            |
| Congo red.....             | Grübler     |            |
| Cresol red.....            | La Motte    |            |
| Crocein.....               | NAC Co.     | 2729       |
| Eosin W.....               | Grübler     |            |
| Erythrosine.....           | B & L Co.** |            |
| Iodine green.....          | NAC Co.     | 4155       |
| Light green SF.....        | PC Co.      |            |
| Magdala red (Phloxin)..... | Grübler     |            |
| Martius yellow.....        | NAC Co.     | NYm        |

\* PC Co. = Providence Chemical Co.

\*\* B and L Co. = Bausch and Lomb Co.

GROUP III DYES—*Concluded*

| Dye                                    | Maker     | Lot number |
|--|-----------|------------|
| Methyl blue.....                       | B & L Co. |            |
| Methyl green B.....                    | NAC Co.   | 2797       |
| Methyl orange.....                     | "         | 2062       |
| Nigrosine (H <sub>2</sub> O sol.)..... | "         | 2685*      |
| Orange I.....                          | "         | 1321       |
| Orange II.....                         | "         | NOb-1      |
| Orange IV.....                         | "         | 1311       |
| Orange G.....                          | Grübler   |            |
| Orcein.....                            | NAC Co.   | 1184       |
| Pbenol red.....                        | La Motte  |            |
| Purpurin.....                          | PC Co.    |            |
| Thymol blue.....                       | LaMotte   |            |
| Trypan blue (vital).....               | NAC Co.   | 2102       |
| Trypan red (vital).....                | "         | 3586       |

None of the Group III dyes accumulate in, or visibly stain, the normal living cells of the cambium and its derivative tissues, regardless of the pH of the buffers. Even after many hours in highly concentrated solutions of the dyes, no color can be detected in the vacuoles of the living tissue cells. *Staining of the vacuoles, as of the protoplasm and nucleus, occurs in irreversibly injured, dying, and dead cells only.* In concentrations of .02 per cent-.5 per cent many of the Group III dyes are less toxic than are Group I and II dyes in dilutions of .004 per cent. Not infrequently the cells live longer than they do in the dye-free buffers used as controls. This is due, at least in part, to the fact that the dyes tend to retard the development of contaminating microorganisms.

## DISCUSSION

Before attempting to discuss the significance of these data, it is essential to consider the effects of a number of variables which may complicate or obscure the problem. No sound generalizations concerning ranges, rates, and intensities of vital staining can be drawn from highly toxic dyes which penetrate slowly and erratically. The staining of cell walls varies markedly not only from dye to dye but also in different ranges of the buffered solutions. Rapid and intense staining of the cell walls may seriously affect the visibility of the vacuoles as well as the accumulation of the dye within them. Where the cells remain in the buffers for some time, the composition and pH

of the vacuolar sap may be modified. Not infrequently the solubility and stability of the dyes vary in phthalates, phosphates, and borates, or in different ranges of pH. In other words, the concentration of the dye in the buffered solutions may be modified through precipitation, crystallization, etc. In certain cases, phenomena of oxidation-reduction may introduce serious complications. A number of the dyes actually are mixtures of different dyes.

The striking differences in the behavior of the Group I and Group II dyes, illustrated in Fig. 2, are not due primarily to the effects of such factors as these. The dyes are not excessively toxic. They penetrate rapidly and uniformly throughout the sections. The phenomena of vital staining are not obscured or seriously modified by an unduly rapid and intense staining of the cell walls. The more rapid accumulation of the Group I dyes in alkaline buffers, and of certain of the Group II dyes in acid buffers, is not due to a greater solubility or stability of the dyes in these buffers. Neutral red and methyl red are less soluble and stable in those ranges of buffers where they accumulate most rapidly and intensely. Nor is the absence of vital staining in certain buffers due to the leaching away of the contents of the vacuoles. Cells which have remained unstained for 24 hours or more in acid solutions of neutral red accumulate the dye rapidly when transferred to alkaline buffers. Similarly, vacuoles which do not stain in alkaline solutions of methyl red accumulate the dye rapidly when transferred to acid buffers. Furthermore, the salient correlations between vital staining and extracellular pH are not due to specific combinations of physico-chemical factors in the Clark buffers. Similar differences in the staining reactions between Group I and Group II dyes occur in solutions prepared with various acids and alkalis.

The most significant of the writers' data in any general discussion of vital staining are accordingly the following:

1. There are two distinct categories of vacuoles in the higher plants, which may be designated, for the time being, as A-type and B-type.

2. Group I dyes vitally stain both categories of vacuoles. These dyes accumulate more rapidly and intensely from alkaline than from acid buffers. The B-type vacuoles rarely, if ever, stain vitally in solutions more acid than pH 5.4–pH 5.8.

3. Group II dyes accumulate in the A-type vacuoles only. The

ranges of pH in which these dyes stain most rapidly and intensely are variable. Certain of the dyes accumulate relatively uniformly throughout a wide range of acid and alkaline buffers. Others stain rapidly and intensely in acid solutions; slowly, if at all, in alkaline ones. No dye which accumulates rapidly from solutions more acid than pH 5.4–pH 5.8 stains the B-type vacuoles. The difference in the behavior of the two categories of vacuoles is not due to a failure of the dyes to penetrate cells which contain B-type vacuoles. This is clearly shown by the fact that *where both types of vacuoles occur within the same streaming protoplast, the A-type stain, whereas the B-type do not.*

4. Group III dyes do not accumulate in either type of vacuole—regardless of the pH of the aqueous solutions in which the dyes are dissolved—unless the tissue cells are irreversibly injured.

5. The A-type vacuoles contain certain categories of aromatic compounds,—e.g., tannins, which tend, when Group I and Group II dyes attain a certain concentration within them, to form copious deeply-colored precipitates. The typical B-type vacuoles do not contain microscopically detectable quantities of such substances and rarely form massive intravacuolar precipitates.

There are two important aspects of the problem of vital staining: (1) penetration of the dye and (2) accumulation of the dye. Among the host of speculations regarding cell permeability the lipid theory has received the most serious and sustained consideration. In so far as the behavior of dyes is concerned, critics of Overton's theory have searched, on the one hand, for lipid-soluble dyes which do not stain vitally and, on the other hand, for lipid-insoluble dyes which do penetrate living cells. Although there appear to be serious sources of error in certain of these investigations,<sup>9</sup> the correlations between solubilities in specific lipoids and vital staining are not sufficiently close to justify the acceptance of Overton's (11) theory in its original form.

Nirenstein (10) attempted to show that there is a close correlation

<sup>9</sup> The criteria for distinguishing irreversibly injured, dying, and dead cells are unreliable. The assumption that absence of vital staining is indicative of failure to penetrate is unsound. Rhodamine, for example, is cited as a lipid soluble dye which penetrates living cells with great difficulty. This dye does not accumulate in B-type vacuoles, but it stains A-type vacuoles the most rapidly and uniformly of any of the dyes tested by the writers.

between vital staining and the partitioning of basic dyes between olive oil plus oleic acid and water, of acid dyes between olive oil plus diamylamine and water, and of all dyes between olive oil plus oleic acid plus diamylamine and water. More recently, Miss Irwin (6; 7) has studied the correlations between the penetration of certain dyes and their partitioning between chloroform and buffers of varying pH.

In view of the fact that the partition coefficient of a dye frequently varies markedly with varying hydrogen ion concentration, it seemed advisable to the writers to supplement their investigation of the effects of extracellular pH upon vital staining by a study of the partitioning of dyes between the buffered solutions and various non-polar solvents.

#### *Partition of Dyes between Buffered Solutions and Non-Polar Solvents*

No attempt was made to secure accurate quantitative determinations of the partition coefficients. The relative amount of dye in each solvent was estimated colorimetrically. The following non-polar solvents were tested, singly and in various mixtures: olive oil, oleic acid, chloroform, carbon tetrachloride, and medicinal liquid petrolatum.

There is an obvious correlation between the accumulation of dyes in vacuoles from buffered solutions and the amount of the dyes that can be extracted from the buffers by chloroform. Thus, the Group III dyes<sup>10</sup> are not extracted in visible quantities by chloroform. The dyes in Groups I and II, which stain more rapidly in alkaline solutions, pass into the chloroform, leaving the alkaline buffers nearly colorless, but are partitioned between acid buffers and chloroform. Such dyes as methyl red, which accumulate in the vacuoles most rapidly from acid buffers, are extracted by chloroform from acid buffers, but are only partially removed from alkaline ones. Rhodamine passes completely into chloroform throughout the pH range. Nile blue BB and safranin bluish are most concentrated in the non-polar solvent at those ranges of pH where the dyes stain best.<sup>11</sup>

<sup>10</sup> Thymol blue is chloroform-soluble at pH 3.0. Unfortunately, vital staining by a slowly penetrating dye cannot be tested at this pH owing to lethal factors.

<sup>11</sup> It should be noted in this connection that the passage of an increasing amount of dye from an aqueous to a non-polar solvent, concomitant with a change of pH, may be due not to an increase in the dye's non-polar solubility but to a decrease in its solubility in the buffer.

Although none of the dyes stain vitally in buffers from which they are not extracted in visible quantities by chloroform, the fact that a dye passes into the non-polar solvent from a specific buffer is no guarantee that the dye will stain vitally at this pH. Crystal violet, for example, at pH 4.6 passes completely from the buffer into the chloroform, but does not stain the vacuoles at this pH.<sup>12</sup> Galloxyaniline and gallamine blue also are extracted from acid buffers by chloroform, although the dyes stain extremely slowly in such buffers.

There is a much closer correlation between the vital staining ranges of many of the dyes and the ranges at which they can pass from the buffers into carbon tetrachloride. In certain cases, the correspondence is very close. Thus, neutral red and chrysoidin Y are partitioned between the buffers and carbon tetrachloride at the pH range where they vitally stain the vacuoles, a greater portion of the dye passing into the non-polar solvent at that part of the range where the staining is most rapid. Certain dyes—rhodamine, crystal violet, celestin blue, janus green B—are more or less insoluble in pure carbon tetrachloride, but they may be rendered soluble by the addition of a trace of oleic acid. Although a mixture of 97 parts of carbon tetrachloride and three parts of oleic acid affords a closer correlation between partition coefficients and details of vital staining than do lipoids, chloroform, or mixtures of olive oil and oleic acid, no single mixture gives equally close correlations with all dyes. It should be emphasized in this connection, however, that the discrepancies are no greater than the differences in the details of vital staining of different plants or tissues.

The more salient correlations between vital staining and the solubilities of the various categories of dyes in non-polar solvents suggest that where dyes penetrate cells readily and rapidly, they are trapped by the A-type vacuoles, whereas the B-type vacuoles may or may not be able to accumulate them. At least, such an assumption provides a working hypothesis which may be tested by subsequent investigations.

It may be objected that A-type vacuoles are unable to trap Group III dyes,—*i.e.*, the dyes penetrate but do not accumulate in the vacuoles. There is no reliable evidence to indicate that the dyes

<sup>12</sup> In a recent paper, Miss Irwin (8) accounts for this behavior of crystal violet upon the basis of "multiple partition coefficients."



penetrate normal living cells of the cambium or of its derivative tissues, but the vacuoles of irreversibly injured, dying, and dead cells may accumulate the dyes and may even form deeply-colored precipitates. Collander's (3) technique of prolonged treatment with highly concentrated solutions yields positive results only with cells whose permeability appears to have been modified by irreversible injuries or incipient cytolysis.

Much has been written concerning the non-polar solubilities and penetration of basic as contrasted with acid dyes. The Group I dyes are all basic dyes. The Group III dyes, on the contrary, are prevailingly acid. Two supposedly basic dyes, iodine green and methyl green B, are included in Group III. These highly methylated triamino-triphenyl-methanes are unstable and readily decompose into other dyes. Until pure samples of these dyes are thoroughly investigated, their inclusion in Group III is of doubtful significance. Group II contains several dyes which have a carboxyl radical,—*i.e.*, methyl red, ethyl red, propyl red, gallocyanine and rhodamine. These dyes pass readily from acid buffers into both non-polar solvents and living tissue cells. Methyl red is listed by Conn (4) as weakly acid, and rhodamine and gallocyanine as basic. According to Kolthoff (9) the free methyl red may be regarded as an ampholytic ion,  $+HN(CH_3)_2-RCOO^-$ . It reacts with acids to form the free carbonylic acid which is a cation,  $+HN(CH_3)_2RCOOH$ . The yellow form of the dye which is an anion,  $N(CH_3)_2RCOO^-$ , predominates in alkaline solutions. This raises the question whether ethyl red, propyl red, gallocyanine, and rhodamine, and such dyes as brilliant green, celestin blue, and gallamine blue are amphoteric.

### *Intracellular pH*

Certain investigators are of the opinion that dyes penetrate *Valonia* and *Nitella* readily in the form of chloroform-soluble free bases, but slowly, if at all, as salts or ions which are relatively insoluble in chloroform. Therefore, the dyes penetrate most rapidly where they exist largely as free bases,—*i.e.*, in alkaline solutions. The dyes accumulate in the vacuoles because the pH of the sap is such that a large proportion of the penetrating free base is converted into the chloroform-insoluble forms of the dye.

Although the vital staining of B-type vacuoles by Group I dyes appears to harmonize with such conceptions regarding penetration and accumulation, the free base hypothesis must account for the rapid penetration of Group II dyes in acid buffers. The vacuoles of *Valonia* and *Nilella* are apparently of the B-type and, in all probability, may be unable to accumulate dyes which belong in the Group II category. The most favorable materials for the study of Group II dyes are cells which contain both A-type and B-type vacuoles within the same protoplast.

Obviously the pH of the vacuoles deserves serious consideration in any discussion of vital staining. The Group I dyes, with the exception of auramin, methylene blue, and methylene green, stain the two categories of vacuoles conspicuously different colors. For example, as stated in the introduction, neutral red stains the A-type vacuoles bluish-magenta, the B-type reddish-orange. The vacuoles stain their respective colors regardless of the pH of the buffers in which the dye is dissolved, and retain their characteristic colors for many hours. Neutral red is one of Sørensen's standard hydrogen ion indicators and has been used extensively in the study of intracellular pH. The certified brand, NX-1, in the dilutions employed by the writers, is orange at pH 8.0, magenta at pH 6.8, and blue in extremely acid solutions. Between pH 7.0 and pH 7.8, it is orange-red or reddish-orange; between pH 5.8 and its "subsidiary H-exponent," bluish-magenta or violet. This suggests that the B-type vacuoles are slightly alkaline and the A-type markedly acid.

In the case of the A-type vacuoles, different indicators (Group I and Group II dyes) give similar values, and the vacuolar sap appears to be markedly acid. In the case of the B-type vacuoles, on the contrary, neutral red may indicate a value of pH 7.2 when brilliant cresyl blue gives a value of approximately pH 13.0. In other words, the various Group I dyes indicate more or less widely separated pH values for the B-type vacuoles, and the "Range Indicator Method" cannot be relied upon in studying the hydrogen ion concentration of such vacuoles.

The thionins and oxazines of Group I are poor indicators. Their color transformations occur in highly alkaline solutions where the dyes are relatively unstable. It may be argued, accordingly, that

neutral red gives a fairly close approximation of the true pH of the B-vacuoles. It should be emphasized in this connection, however, that the vacuoles of *Nitella* are of the B-type. The pH of the expressed sap has been determined, both colorimetrically and electrometrically, as close to 5.6, but, when vitally stained by neutral red, the vacuoles have an apparent pH of from 7.0 to 7.8. This suggests that all of the Group I dyes give pH values for the B-type vacuoles that are excessively alkaline. The discrepancies appear to be larger than those produced by ordinary "salt" errors. The question arises—Are they due to "protein," or "lipoid" errors?

No direct evidence is available, as yet, for answering this question, but certain data obtained in the study of the partitioning of the dyes between buffers and non-polar solvents may prove to be of some significance. When neutral red is partitioned between pure chloroform and the seventeen Clark buffers, the non-polar solvent is orange, or colorless where in contact with the more acid buffers. When a trace  $\frac{(M)}{(120)}$  of oleic acid is added to the chloroform, the color of the non-polar solvent is magenta where in contact with buffers more acid than pH 5.8, and reddish-orange where in contact with buffers more alkaline than pH 5.8. The oleic acid saponifies rapidly between pH 9.0 and pH 10.0. When ordinary commercial chloroform is used, or where a trace of hydrochloric acid is added to pure chloroform, the non-polar solvent is orange where in contact with buffers more alkaline than pH 6.2, magenta where in contact with buffers more acid than pH 5.0, and reddish-orange where in contact with the much restricted range of intermediate buffers.<sup>13</sup> In other words, when neutral red is partitioned between commercial chloroform and Clark buffers, the virage of the B-vacuoles matches that of the pH 7.2 buffer and of the chloroform in contact with the pH 5.8 buffer. As previously stated, there is a similar discrepancy in *Nitella* between the true pH and the neutral red virage. This might well be brushed aside as a mere coincidence

<sup>13</sup> That the pH of the  $\frac{M}{20}$  buffers is not markedly altered may be determined by comparing the virages of the buffers before and after shaking with the non-polar solvents. The buffers and non-polar solvents were tested in various ratios, volumes of 5:1, 3:1, 1:1, etc.

were it not for the fact that in the case of all of the Group I dyes the virage of the chloroform in contact with the pH 5.8 buffer matches the virage of the B-type vacuoles. The possibility that the vacuoles of this category contain some substance or combination of substances<sup>14</sup> that produce excessively alkaline virages with basic dye indicators deserves serious consideration.

That the pH of the B-type vacuoles may be between 5.4 and 6.0 is suggested not only by *Nitella*, but also by certain reactions of these vacuoles in vital staining. In the writers' experiments the B-type vacuoles rarely, if ever, stain below pH 5.4–pH 5.8. Furthermore, no dye which penetrates readily in solutions more acid than pH 5.4–pH 5.8 accumulates in the B-type vacuoles. If the pH of these vacuoles is approximately 5.6, the accumulation of Group I dyes may be accounted for, as in *Valonia* and *Nitella*, by the assumption that the dyes penetrate the cell faster from alkaline buffers than they escape from the relatively acid vacuoles. The failure of the Group II dyes to accumulate in the B-type vacuoles may be due to the fact that these dyes penetrate the protoplast so readily from acid solutions that they escape from pH 5.6 vacuoles as rapidly as they enter. It may be significant, in addition, that streaming cells which contain B-type vacuoles tend to survive the longest in the pH 5.0–pH 6.2 buffers.

### *Contents of Vacuoles*

As previously stated, the A-type vacuoles contain certain categories of aromatic compounds,—e.g., tannins, tanniferous glucosides, natural pigments, etc., and where Group I dyes and Group II dyes penetrate the cells, they tend to be trapped by entering into physico-chemical combinations with the complex colloidal contents of the vacuoles. The B-type vacuoles, on the contrary, do not contain microscopically detectable quantities of such aromatic compounds, and they do not accumulate Group II dyes, or Group I dyes from

<sup>14</sup> Much controversy has arisen among cytologists concerning the presence of fats or lipoids in the vacuole. Although the cumulative evidence which suggests that they are present is rather tenuous, their absence cannot be demonstrated by testing with Sudan III and similar microchemical tests for fats and lipoids. The hypothetical substances may well exist in such forms or quantities that they cannot be detected by relatively crude microchemical techniques.

buffers more acid than pH 5.4 to pH 5.8. It should be noted; however, that the B-type vacuoles of certain species and tissues do form colored granules, Liesegang rings, and spherocrystals, and that the intensity of vital staining varies markedly from tissue to tissue. This suggests that the B-type vacuoles may contain more or less "tannin"-free colloidal material and that, where Group I dyes accumulate in the vacuoles, a portion of the dye may be held in physico-chemical combinations, at least during the later stages of vital staining.

Many investigators have argued that active proteins or other ampholytes play an important rôle in the penetration and accumulation of dyes. The Dangeards (5) maintain that all plant vacuoles contain metachromatin or similar colloidal nitrogenous substances—either with or without adsorbed phenolic compounds—which stain readily with basic vital dyes. The formation of colored precipitates and the intensity of vital staining of the vacuoles are considered to vary with different degrees of hydration and dehydration of these colloidal substances. Scarth (12) holds that "hydrated lipoids" are concerned in the accumulation of dyes in both the vacuoles and the cell walls of living cells. The writers' data suggest that the B-type vacuoles contain some substance or mixture of substances that produce huge errors in colorimetric determinations of the pH of the vacuolar sap.

Although the accumulation of Group I dyes in the vacuoles of *Nitella* and *Valonia* may be due, primarily, to differences in the pH of the intracellular and extracellular solutions, it cannot wisely be inferred that all B-type vacuoles function in a similar manner. In certain categories of B-vacuoles, as in A-type vacuoles, the operation of a pH trap may be modified or replaced by that of a colloidal trap. Further speculation concerning the matter is best deferred until more reliable data concerning the contents and pH of B-type vacuoles are available.

#### CONCLUSION

It should be emphasized, in conclusion, that the writers' investigation is a reconnaissance, and was initiated primarily in searching for more adequate techniques for the study of cytological problems. Crude as many of the data undoubtedly are, they are of some significance in outlining future trends of more intensive investigation. The occurrence of two distinct types of vacuoles within the same cell provides

a valuable check upon generalizations concerning the penetration of certain dyes. The A-type vacuole affords a means of determining that a number of dyes do penetrate living plant cells readily and rapidly from acid buffers. The recognition of two distinct categories of vacuoles—which are widely distributed throughout the higher plants—and a study of their staining reactions in Group I, Group II, and Group III dyes, indicate that certain discrepancies in the literature are due to the fact that different investigators are concerned with different vacuoles and with different dyes. For an accurate visualization of the physico-chemical mechanisms of the penetration and accumulation of dyes in living cells a much wider range of reliable data is essential, both as regards the purely biological variables and the physico-chemical variables in techniques employed in their investigation. Until such data are available, generalizations from limited induction should be reduced to a minimum.

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This is followed by an upward movement (which we will call *b*, Figs. 2 and 3) the start of which seems to be practically simultaneous at both places but the upward movement of the string is slower at *B* than at *A* since the width of the white line where the curve starts upward<sup>4</sup> is greater in the lower curve than in the upper.

The upward movement is followed by a downward movement (which we will call *c*) starting later at *B* than at *A*. The next move-

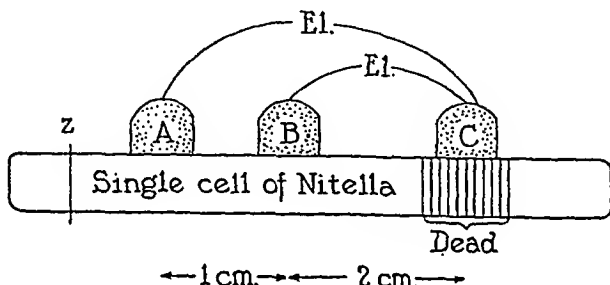


FIG. 1. Diagram to show arrangement of experiments. At *A* and *B* 0.001 *M* KCl is applied. The protoplasm at *C* is killed by applying 0.001 *M* KCl saturated with chloroform and the cell is cut at *Z*. The resulting response is shown diagrammatically in Fig. 2.

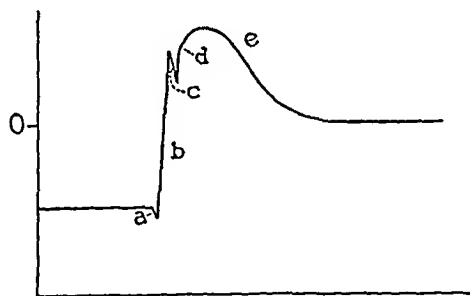


FIG. 2. Diagram to show the electrical responses at *A* due to cutting at *Z* (cf. Fig. 1). The duration of movements *a*, *b*, and *c* is exaggerated.

ment is upward, marked *d* in the slower record shown in Fig. 4 (*a* and *b* cannot be seen in this slow record, but *c* can be made out by close inspection in the upper curve and is very clear in the lower one).

<sup>4</sup> When the experiment is arranged as in Fig. 1 but with *C* alive, a complication is sometimes observed when *C* is close to the end of the cell. In such cases transmission to *C* appears slow and the curves have various irregular shapes.

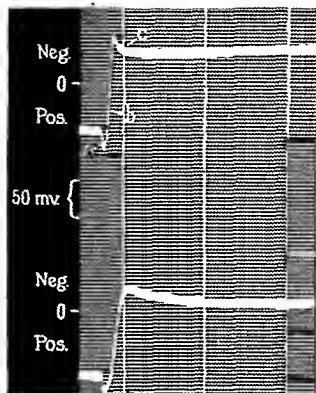


FIG. 3. Photographic record showing the result of an experiment arranged as in Fig. 1. The upper curve records the r.d. of *A* with reference to *C*; the lower curve that of *B* with reference to *C*. Cutting the cell at *Z* produces the movements *a*, *b*, and *c*. The distances between vertical marks represent intervals of one-tenth of a second. At the same time a slower record (Fig. 4) is taken on another galvanometer.

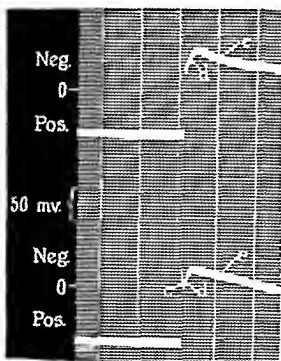


FIG. 4. Slower record of the same experiment as that shown in Fig. 3 (taken simultaneously on another galvanometer). The distances between vertical lines represent 5-second intervals.



This is followed by a downward movement (*e*) which ends by approaching zero.<sup>5</sup>

It is evident from Fig. 3 that the *c* movement starts later at *B* (lower curve) than at *A* (upper curve) and this difference in time will for convenience be called the lag. In general the lag appears to increase with the successive movements, *i.e.* it is greater for *d* than for *c* and still greater for *e*. The lag also appears to increase as the mechanical disturbance travels down the cell. For example, measurements on 15 cells showed that the lag of the *c* movement between *A* and *B*

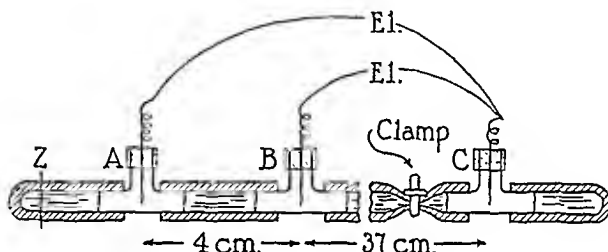


FIG. 5. Diagram of an experiment on a piece of soft rubber tubing with Ag-AgCl electrodes in glass T-tubes at *A*, *B*, and *C*. The tube is filled with tap water (all air bubbles being excluded). Between *B* and *C* the tube is clamped to prevent movement of water at *C*. In some experiments a U-tube filled with sand was substituted for the clamp. The tube was wound at *Z* with copper wire which was connected to the striking hammer to keep the two at the same potential and avoid communicating a charge.

(1 cm. apart)<sup>6</sup> was 0.008 sec. and between *B* and *D* (situated 1 cm. to the right of *B*) was 0.043 sec.

<sup>5</sup> This is clearly shown in previous papers.<sup>1</sup>

Certain of these movements may be lacking and the curve may have only one crest.

In cutting *Chara coronata* and *Nitella flexilis* it was sometimes observed that when the knife remained in contact with the cut so that no sap flowed out no death wave occurred but only a response resembling a negative variation and a second one could be produced by cutting the same cell again. These sometimes traveled with the speed of the death wave, and sometimes with the speed of the negative variation. Such responses will be discussed in a subsequent paper.

<sup>6</sup> The distance from the right edge of contact *A* to the left edge of contact *B* is 1 cm. This distance is taken because if an electrical impulse travels from

The facts indicate that the mechanical disturbance loses intensity as it proceeds so that the reactions it starts at each successive spot it reaches proceed the more slowly the greater the distance from the cut.

To learn something of the nature of these electrical changes experi-

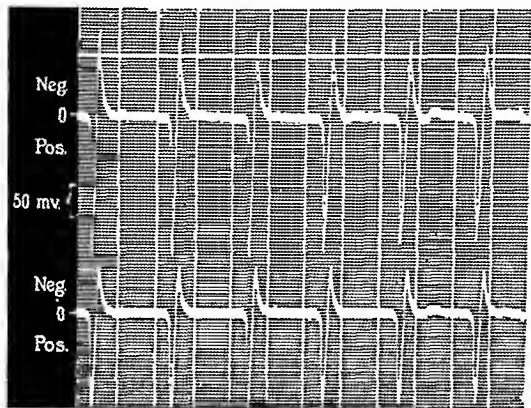


FIG. 6. Photographic record showing electrical responses obtained in an experiment with a soft rubber tube arranged as in Fig. 5. The upper curve records the potential of *A* with reference to *C* and the lower curve that of *B* with reference to *C*. The tube was struck 6 times at *Z*. Each blow produced electrical responses due to the movement of the tap water past the electrodes. The distances between vertical marks represent one-tenth seconds.

ments were made with a rubber tube,<sup>7</sup> inserting glass T-tubes containing Ag-AgCl electrodes about 4 cm. apart,<sup>8</sup> connected to a third electrode (*C*) as shown in Fig. 5. In order to prevent any response in *C*

left to right it registers at each contact as soon as it reaches the left edge of the contact, and the time required for transmission to the right edge of the contact is negligible.

<sup>7</sup> An ordinary soft red rubber tube of about 3 mm. internal diameter.

<sup>8</sup> The electrodes were separated by 2 cm. of glass tubing and 2 cm. of rubber tubing.

it was placed 37 cm. away and a clamp<sup>9</sup> was inserted to prevent the compression wave from reaching it. On striking the tube at *Z* a record was obtained as shown in Fig. 6. Here we find a downward followed by an upward movement and a return to zero. These appear to be practically simultaneous at *A* and *B* and may be comparable with movements *a* and *b* in the living cell.<sup>10</sup> They may be regarded as due to the movement of liquid (streaming potential?) past the electrodes in the case of the tube or in the case of the cell past protoplasmic surfaces in which little or no alteration has occurred (the protoplasmic surface need not be solid). In both cases the electrical response falls off as the mechanical disturbance decreases.

Do the slower movements (*c*, *d*, and *e*) in the living cell arise from factors not present in the rubber tubing? They might be interpreted as the result of changes in the protoplasm, either structural or due to the inrush of ions into the protoplasm.<sup>11</sup> These changes in the protoplasm seem to proceed at different rates in its outer and inner surfaces since the form of the curve changes decidedly<sup>12</sup> when the solution applied to the outside of the cell is altered (which is not true of the fast movements *a* and *b*). The form of the curve has been discussed in previous papers:<sup>1</sup> it changes with distance from the cut in a manner which suggests that the intensity of the mechanical disturbance falls off as it travels.

It might be suggested that the slower movements are connected with an outflow of sap resulting from the cut but these movements are sometimes found when the cell is vigorously bent or pinched<sup>13</sup> without producing an outflow of sap.

<sup>9</sup> The clamp did not introduce sufficient resistance to interfere with the electrical measurement, since the resistance of the measuring instrument is over 30 megohms.

<sup>10</sup> When these electrodes were 40 cm. apart the rate of transmission was found to be about 80 meters per second.

<sup>11</sup> Osterhout, W. J. V., and Hill, S. E., *J. Gen. Physiol.*, 1929-30, 13, 459, 547. Osterhout, W. J. V., Electrical phenomena in the living cell, in Harvey Lectures, 1929-30, The Williams and Wilkins Co., Baltimore, 1931, 169. Blinks, L. R., *J. Gen. Physiol.*, 1930-31, 14, 127.

<sup>12</sup> When 0.001 M KCl is outside, the *d* movement is negative (upward); when 0.1 M KCl is outside, the *d* movement is positive (downward). Osterhout, W. J. V., and Harris, E. S., *J. Gen. Physiol.*, 1928-29, 12, 167, 355.

<sup>13</sup> This will be discussed in a subsequent paper. A vigorous bend or pinch (with no outflow of sap) may produce a true death wave.

The electrical disturbance may pass to a neighboring cell as noted by Jost,<sup>14</sup> but the question arises whether the electrical response in the second cell is a death wave or a negative variation. A death wave might possibly result from a mechanical disturbance in the second cell but if this were lacking a negative variation might start as the result of (1) a mechanical disturbance insufficient to produce a death wave, or (2) a flow of current between the first and second cells, as elsewhere discussed.<sup>15</sup>

Some of the differences between death waves<sup>16</sup> and negative variations may be considered in this connection.

1. The death wave can pass a killed spot while the negative variation cannot except by the aid of a salt bridge.<sup>15</sup> On this basis the responses in the second cell must be regarded as negative variations, as shown by experiments in which a spot was killed in the middle of the second cell.

2. The death wave always shows a marked decrement in the intensity and speed of the response as it travels along: since this is not true of the response in the second cell it would appear to be a negative variation. The decrement shown by the death wave must be due to the fact that the mechanical disturbance becomes less intense as it travels along the cell and that the protoplasmic response falls off<sup>1</sup> as the intensity of the mechanical disturbance diminishes.<sup>17</sup>

3. Leading off near the cut from a spot in contact with 0.001 M KCl or tap water we observe a change in the sign of the P.D. across the

<sup>14</sup> Jost, L., *Sitzungsber. Heidelberger Akad. Wissensch. Math.-naturw. Klasse*, 1927, Abhandl. 13. Jost reports that the stimulus due to cutting may pass to a second, third, or a fourth cell. With our technique we have not seen any response (death wave or negative variation) in a third or fourth cell. When we used his technique we apparently got the result reported by Jost, but we interpret it somewhat differently. We suppose that when all the cells but one in the chain (in their natural union) are in the same reservoir of tap water a cut in any cell in the reservoir may be registered by the electrometer whether it causes a response in a neighboring cell or not.

<sup>15</sup> Osterhout, W. J. V., and Hill, S. E., *J. Gen. Physiol.*, 1929-30, 13, 547.

<sup>16</sup> It will be shown in a subsequent paper that pinching or bending may produce responses intermediate in character between death waves and negative variations.

<sup>17</sup> Not only the amplitude but the speed of the response falls off, as mentioned above.

protoplasm when the cut is made:<sup>1</sup> if the spot is far enough away from the cut this does not occur but the curve goes to zero. In either case there is no recovery (*i.e.* the curve does not return to its original position). The response in the second cell does not show a change of sign and does show recovery.

4. The apparent speed of transmission of all the movements of the death wave is much greater than that of the negative variation. On this ground also the response in the second cell seems to be a negative variation since it is only about 2 cm. per second.

It would therefore seem that the response in the second cell is not a death wave but it is not necessary to conclude that it is due to a flow of current between the two cells (as elsewhere discussed<sup>1b</sup>), since this seldom occurs in the absence of a salt bridge. It seems more probable that a mechanical impulse reaches the second cell and produces an electrical disturbance which continues down the cell as a propagated negative variation. This will be discussed in a subsequent paper.

#### SUMMARY

Cutting a cell of *Nitella* sets up a series of rapid electrical responses, transmitted at a rate too rapid to be measured by means of our records. These are followed by slower responses whose speed falls off as the distance from the cut increases, as though they were caused by a mechanical disturbance whose intensity falls off as it travels.

The faster responses seem to be due to the motion of sap past protoplasmic surfaces which have suffered little or no alteration (they seem to be similar to the electrical changes following a blow on the end of a soft rubber tube containing Ag-AgCl electrodes). The slower responses appear to be due to alterations in the protoplasm and are usually irreversible.

# THE OSMOTIC PROPERTIES OF LIVING CELLS (EGGS OF *ARBACIA PUNCTULATA*)

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When a cell such as the unfertilized egg of the sea urchin *Arbacia punctulata* is placed in a solution hypotonic with respect to its natural medium, water enters and the cell swells; conversely, it shrinks in hypertonic solutions. This observation at once suggests that the force which causes water to enter is osmotic pressure, that the cell is acting as an osmometer. Earlier measurements of cells in equilibrium with anisotonic solutions indicated that the unfertilized *Arbacia* egg is an osmometer of a high degree of excellence, although the observed volume of cells at equilibrium fell somewhat short of the calculated value (1). Since the relations between pressure and volume of the cell forms the basis for a kinetic theory of osmosis which will be given in a subsequent paper (2), it was necessary to determine this relation as accurately as possible, by means of further experiments.

The present paper therefore attempts to answer the question: how nearly ideal in its osmotic properties is the *Arbacia* egg?

An ideal osmometer should meet the following requirements: (1) One should be able to measure volume accurately at all pressures. (2) The product of pressure and volume should be constant (law of Boyle-Van't Hoff). (3) The membrane should be actually semi-permeable, *i.e.*, should allow passage of water, but not of dissolved substances. (4) The transport of water should be due to osmotic force alone.

These requirements will be taken up in order.

## 1. Measurement of Cell Volume

One of the greatest advantages of the *Arbacia* egg, as compared with other cells used as natural osmometers, is its generally spherical

shape. This fact, together with its relatively large size, allows direct and accurate measurement of diameter (and so of volume) with microscope and ocular micrometer.

In measuring, only optically round cells—these constitute the great majority—are selected. When these are rolled over under the microscope they are seen to be spherical in fact.

However there remains the possibility that the eggs might flatten under their own weight as they rest on the bottom of the dish.\* If such flattening take place, the measurement of the horizontal optical section would cause the volume to be overestimated, and our data would need to be corrected.

The simultaneous measurement of horizontal and vertical diameters of an egg should present no serious difficulties. However, we have employed an even simpler method for detecting the flattening of eggs under their own weight. This method consists in placing a sample of eggs in a water-tight chamber, whose top and bottom are formed of cover-slips. After settling, the diameters of 50 eggs are measured, in the ordinary way. The chamber is now gently turned over; with the exception of a very small percentage the eggs will stick to the surface on which they were originally resting, and so come to hang from what is now the top of the chamber. The same force which tended to flatten the eggs as they rested on the bottom now tends to elongate them. The horizontal diameters of approximately the same 50 eggs may again be measured; if there should be any tendency for the eggs to become deformed under the action of their weight, it would be detected in a diminution of the diameter of the optical section. Moreover, the effect would be (approximately) doubled.

In order to facilitate the sticking of the eggs it is advisable first to coat the cover-slips with a thin film of albumin—especially when eggs are in equilibrium with the more hypotonic solutions, as in these solutions the natural stickiness of the eggs is considerably diminished.

Table I gives the results of several such experiments. The average volume of 50 cells is used in each case; in the first column is the concentration of sea water in which the eggs are in equilibrium; in the second column is the average volume computed from measurements

\* Vlès (3) has reported that flattening occurs in the eggs of another species of sea urchin.

made with the eggs resting on the bottom of the chamber; in the last column is the average volume of approximately the same 50 eggs, computed from measurements of diameter made with the chamber inverted and the eggs hanging from the top.

It is seen that the volumes of resting and hanging eggs agree very closely. We conclude that in these experiments there is no deformation of the eggs, under the action of their weight.

There remains the possibility of a semipermanent deformation, which requires an appreciable time to become established. In that

TABLE I

Experiment to determine whether cells are flattened. In Column 1 is given the concentration of sea water with which cells are in equilibrium. Columns 2 and 3 give volume of cells resting on the bottom of the chamber and suspended from the top, respectively.

Each figure represents the mean of 50 cells. These figures must be multiplied by 100 to obtain actual volumes in cubic micra.

The figures indicate that these cells are not appreciably flattened either in isotonic or hypotonic sea water.

| Concentration of sea water<br><i>per cent</i> | Cell volumes      |                |
|---|-------------------|----------------|
|   | Bottom of chamber | Top of chamber |
| 100   | 2015              | 2015           |
| 60  | 3184              | 3184           |
| 60  | 3162              | 3195           |
| 50  | 3900              | 3924           |
| 50  | 3814              | 3850           |

case our measurements would fail to detect the deformation, as they are made within a few minutes of inverting the cell. However, such a semipermanent deformation could readily be detected by rolling the eggs over, under observation. Measurements made in this way on a number of diameters of the egg have failed to show any appreciable lack of sphericity.

We may thus conclude that the eggs of this species are not appreciably deformed in any of the solutions used in these experiments, under the action of their weight in these media.



## 2. *Application of the Boyle-Van't Hoff Law*

In the theory of the ideal, dilute solution, the osmotic pressure of a given amount of dissolved substance is inversely proportional to the volume in which it is distributed. The assumption that the solutions of the present system behave as ideal, dilute ones is an approximation which permits the application of the simple laws governing osmotic processes. An attempt to evaluate the activities of all the constituents of the cell and its medium is of course out of the question.

We may, therefore, write,

$$PV = K \quad (I)$$

where  $P$  is the osmotic pressure of the dissolved substances in the egg,  $V$  is the volume of the egg, and  $K$  is a constant. From the consideration that the egg initially must be in equilibrium with ordinary sea water, of known osmotic pressure, it is seen that  $K$  may be determined experimentally by measuring the volume of the egg as it rests in its normal medium. We write for (I) its equivalent:

$$PV = P_o V_o \quad (I a)$$

where  $P_o$  is the osmotic pressure of ordinary sea water and  $V_o$  the volume of the egg in its normal medium.

This equation furnishes a method for determining, at any time, the osmotic pressure in the interior of the egg. In addition, it furnishes the law that the equilibrium volumes of the egg must follow in anisotonic solutions. In these cases the internal pressure must equal that of the external medium, and the expected volume of the egg at equilibrium is given by

$$P_{ex} V_e = P_o V_o \quad (I b)$$

where  $P_{ex}$  is the osmotic pressure of the external medium (known from its concentration) and  $V_e$  is the expected equilibrium volume of the egg. (I b) therefore furnishes the means of determining whether the Boyle-Van't Hoff law is obeyed by this osmometer.

The earliest measurements of the *Arbacia* egg in equilibrium with hypotonic solutions were made by R. S. Lillie (4). He found that when sea water was diluted two and one-half times the eggs only doubled

in volume. As stated above smaller discrepancies were reported by the present authors in an earlier paper (1).

### *Experimental Method*

Eggs from a single animal were washed and very gently centrifuged. The supernatant fluid was discarded. The remaining heavy suspension of eggs was distributed in 200 cc. of the several dilutions of sea water. Subsequently, the eggs were pipetted into fresh solution contained in Stender dishes. These were kept at room temperature 19°C. Measurements were made from 2 to 5 hours later, after repeated preliminary measurements showed that the cells had reached constant volume.

In two subsequent experiments the cells were stored over night at 10°C. and measured the following morning. Measurements were made in one case at room temperature, in the other at 10 to 12°C.

The optical system employed was a 10 mm. objective (immersed in the sea water) and a filar ocular micrometer, giving a magnification of 240 diameters.

The solutions employed were undiluted sea water (100 per cent), 90 per cent (*i.e.*, 90 parts of sea water with 10 parts of distilled water) 80, 70, 60 and 50 per cent. 50 cells in equilibrium with each of these solutions were measured by each of two observers.

After measurement, cells (in two of the experiments) were returned to ordinary sea water and sperm was added. Normal cleavage took place, except in the case of cells taken from 50 per cent sea water; these showed atypical cleavage.

### RESULTS

The results of three experiments are recorded in Table II.\* In the first column are given relative pressures, in the second observed volumes and in the fifth column, volumes calculated by Equation (I *b*). The combined data are presented graphically in Fig. 1, where the lower curve represents observed volumes of 300 cells plotted against the relative pressure (or concentration) of the outside solution, and the upper curve is calculated by Equation (I *b*). It is seen that there is a slight deviation of the two curves which becomes more marked as we pass from higher to lower pressures. But even in 50 per cent solution the cells fail by less than 6 per cent to attain the volume calculated. Therefore Equation (I *b*) proves to be a rather fair first approximation.

\* The three experiments reported in this paper are selected as probably the most accurate of a large number of determinations made over a period of several years.



The discrepancy, however, is a real one. In Column 3 of Table II are given the pressure volume products of each experiment. In each case it is seen that there is a slight but definite drift in values of  $PV$ .

There are a number of factors which might prevent the cell from swelling as much as predicted. Of these the one that is surely important and which might account for practically the entire discrepancy is that obviously a considerable fraction of the cell volume is occupied

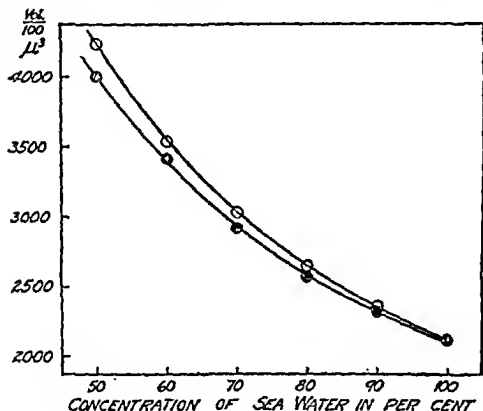


FIG. 1. Comparison of observed and calculated volumes. Solid circles represent mean volumes of 300 cells in osmotic equilibrium with various dilutions of sea water. Open circles are volumes calculated by Equation (Ib). Data are taken from Table II.

by material that can exert little or no osmotic pressure. This fact is best demonstrated by rapidly centrifuging the eggs, whereupon zones of oil and pigment granules separate from the rest of the cytoplasm. The osmotically active solution therefore occupies a volume which is equal not to  $V$  but to  $V - b$ , in which  $b$  is the volume occupied by osmotically inactive material.

Equations (I) to (I *b*) now become, respectively,

$$P (V - b) = K \quad (\text{II})$$

$$P (V - b) = P_o (V_o - b) \quad (\text{II } a)$$

$$P_{ex} (V_c - b) = P_o (V_o - b) \quad (\text{II } b)$$

The value of *b* may be found by substituting in Equation (II *b*) the values given in Columns 1 and 2 of Table II. Since this method of computing gives a considerable scattering of values for *b*, a graphic method is preferred, one in which all points can be weighed equally. Observed volumes are plotted against the reciprocal of the relative pressures, a straight line is fitted to the points and extrapolated to  $\frac{1}{P} = 0$ , and the corresponding volume is read off. In these three experiments, *b* is found to equal 150, 250 and 310 respectively; for the combined data, *b* equals 240, which is 11 per cent of the cell volume in ordinary sea water. This computation therefore indicates that osmotically inactive material occupies in the neighborhood of 11 per cent of the cell volume.\*

In Column 4 of Table II,  $P (V - b)$  has been calculated for these experiments. The resulting values show no definite drift, so that it may be stated that Equation (II) fits the data satisfactorily. The last column of Table II gives volumes calculated by Equation (II *b*). It is seen that these values are in good agreement with observed volumes (Column 2).

These experiments, therefore, show that with this material the Boyle-Van't Hoff law, corrected for volume occupied by osmotically inactive material, holds for pressures of 11 to 22 atmospheres.

It is interesting to compare these conclusions with those of other workers based on different types of isolated cells.

Höfler (5), with certain plant cells containing a large sap vacuole surrounded by an extremely thin layer of protoplasm, found that the pressure-volume product over a certain range of osmotic pressures is constant; that is, *b*, as defined above, is equal to zero.

Ege (6) found that the "disperse phase," *b*, in rabbit erythrocytes is equal to about 40 per cent of the cell volume, while Wieringa (7) reported that in certain yeasts, the volume occupied by osmotically

\* *b* so determined also includes volume of solutes themselves.

inactive material plus the volume of the cell wall equals about 64 per cent of the cell volume.

Making corrections for these values of "*b*," both Ege and Wieringa found close agreement between calculated and observed volumes of cells.

### *3. The Semipermeability of the Membrane*

Of the other factors that might prevent the cell from attaining the equilibrium volume predicted by Equation (I *b*), imperfect semipermeability of the cell membrane at once comes to mind.\* Leakage of salts or other contents from the cell in hypotonic solutions would of course have this effect.

However, it is improbable that such leakage occurs to an appreciable extent in uninjured cells. As shown above, the differences between volumes observed and those calculated by Equation (I *b*) is slight and is satisfactorily accounted for by the presence of osmotically inactive material.

Another type of experiment supports the view that the semipermeability of the membrane is practically perfect. Cells are measured in ordinary sea water, then swollen in a hypotonic solution, returned to ordinary sea water and remeasured after they have come into equilibrium. If cell contents escaped during swelling, it is unlikely (though not impossible) that the first and second measurements should agree. But early experiments (8) indicated that this is the case, that swelling is completely reversible.

Since the question seemed important the experiment was carefully repeated last summer.

In Table III it is seen that the original and final volumes are in good agreement. Thus in the first experiment the original mean volume was  $1864 \times 10^3 \mu^3$ ; after swelling for 1 hour in 50 per cent sea water and shrinking in ordinary sea water the mean volume was  $1853 \times 10^3 \mu^3$ . Cells from the same animal swollen in 60 per cent sea water later returned in ordinary sea water to a volume of  $1874 \times 10^3 \mu^3$ . Similar results were obtained in several other experiments, of which two are included in Table III.

\* It is assumed that the cell surface acts as the membrane of the osmometer.

Thus the weight of evidence is against appreciable leaking of cell contents when the cell is placed in hypotonic solutions, at least over the range of pressures here used. This is true, however, only as long as the cell is uninjured. Injured cells, as has been shown elsewhere (9), lose their semipermeability to a greater or less extent; if loss of semipermeability is marked they behave in hypotonic solutions quite differently from normal cells. In the experiments recorded in Table II, the cells were shown by the fertilization test not to have been injured, except those in 50 per cent solution; these underwent atypical cleavage and therefore were slightly injured.

TABLE III

In these three experiments cells were measured in ordinary sea water (Column 1) and then swollen in 50 per cent sea water for 1 hour. Measurement then showed that they were nearly in equilibrium with this solution. They were returned to ordinary sea water and allowed to shrink for several hours, then remeasured (Column 2).

Similarly cells were swollen in 60 per cent sea water for  $1\frac{1}{2}$  hours, then shrunk and remeasured (Column 3).

It is seen that the cells returned approximately to their original volume.

Each figure represents the mean volume of 50 cells. The temperature was  $15^{\circ}\text{C}$ .

| Original volume | Final volume in ordinary sea water after swelling in: |             |
|-----------------|---|-------------|
|                 | 50 per cent   | 60 per cent |
| 1864            | 1853  | 1874        |
| 2020            | 1994  | 2027        |
| 1823            | 1882  | 1800        |

#### 4. *The Effect of Other Forces on the Equilibrium Volume*

That forces other than osmotic force affect the volume of the cell in equilibrium with its medium, is highly probable. Elasticity and surface tension at once come to mind. But the fact that Equation (II) fits the data indicates that the magnitude of such forces, compared with that of the osmotic driving force, must be quite small.

#### SUMMARY

We have attempted to answer the question: How nearly ideal, as an osmometer, is the unfertilized *Arbacia* egg? The following conclusions have been reached:

1. Volumes can be measured accurately over a wide range of pressures since the cell is in general spherical and does not suffer deformation from its own weight or other factors.

2. The product of volume and pressure is approximately constant, if allowance be made for osmotically inactive cell contents. It is computed that from 7 to 14 per cent of cell volume is occupied by osmotically inactive material.

3. Evidence is presented that no appreciable escape of cell contents occurs while the cell is in hypotonic sea water; that, therefore, the semipermeability of the membrane is approximately perfect, so long as injury to the cell is avoided.

4. In comparison with osmotic pressure the influence of other forces, such as elasticity or surface tension, on cell volume must in these experiments be slight.

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## FURTHER STUDIES ON THE KINETICS OF OSMOSIS IN LIVING CELLS

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In a series of papers (1-5) it has been shown that a study of the kinetics of osmotic swelling and shrinking of the *Arbacia* egg may yield information concerning an important property of the cell—*viz.*, the ease with which water can enter or leave it, under the influence of osmotic forces. This permeability of the cell to water has been shown to be affected by many factors, such as temperature, specificity and valence of ion, etc.

The earliest study of the kinetics of osmotic swelling of cells was made by Lillie (6). He showed that the course of the process may be described by the equation which governs the course of a unimolecular chemical reaction. The velocity constant of the process, given by this equation, was used in our earlier papers as a measure of the permeability of the cell to water. It was found that the "permeability," measured in this way, depended, among other factors, upon the salt concentration of the solution in which the cells were swelling or shrinking (3). Northrop (7), in an investigation of the theory of osmotic swelling, showed that the unimolecular equation was, in this case, devoid of any particular physical significance, and developed an equation which satisfactorily described the course of osmotic swelling in the *Arbacia* egg. Using the data published by us, he investigated the effect of concentration upon permeability (now differently defined) according to his theoretical treatment. This new permeability constant proved to be much less affected by concentration, although, with the data available to him, there was still an appreciable effect.

This reopened the question of effect of salt concentration, and we wished to inquire whether the data indicate a true change in the per-

meability of the cell with changing concentration of the external medium. To this end we have made a more extensive series of experiments. As our attention had been called to the empirical nature of the Lillie equation prior to the appearance of Northrop's paper, and as we had already developed and applied an equation whose derivation differs somewhat from that of Northrop's, we shall include in this paper our theoretical treatment, and apply it, together with Northrop's, to the new data.

### *Theory*

That the *Arbacia* egg constitutes an osmotic system is suggested by the simple observation of its swelling or shrinking when placed in a medium whose osmotic pressure differs from that of sea water. To account for the experimental data quantitatively in terms of the known laws governing osmotic processes, it is necessary to make certain assumptions concerning the cell and its surroundings:

1. The *Arbacia* egg consists of a solution of osmotically active substances, surrounded by a semipermeable membrane.

The fact that a fraction of cell volume is occupied by osmotically inactive material is neglected for the moment for the sake of simplicity, but will be introduced later as a correction.

2. The aqueous systems concerned obey the laws of ideal dilute solutions.

3. Only osmotic forces are concerned in the passage of water across the surface of the egg.

Assumptions 1 to 3 have been discussed in a previous communication (8).

4. The resistance to the diffusion of water is confined to the surface of the egg.

This neglects the time required to equalize concentration differences within the egg itself, as compared with that required for the water to diffuse across the membrane. It is made in the interests of simplicity of mathematical treatment.

5. The resistance to the diffusion of water, per unit area of cell surface, is constant.

This assumption in effect neglects any change in the properties of the membrane as the cell swells or shrinks. In the absence of defi-

nite knowledge concerning the structure of the membrane, this seems to be the most general method of treatment. Northrop (7), however, has dealt with this problem differently; a comparison of the resulting theories will be made in the present paper.

These assumptions permit the development of a theory which shall attempt to account, quantitatively, for the experimental facts of swelling and shrinking. It is evident that when an *Arbacia* egg is placed in a solution the osmotic pressure of which is not equal to that of the interior of the egg, water will be transported across the membrane under the influence of the outstanding difference in pressure on the two sides. This transfer takes place, according to the laws governing such processes, in such a direction as to tend to equalize the difference in pressure—i.e., from the more dilute to the more concentrated medium. The egg, being a closed system, will consequently gain or lose water, and will finally come into a condition of equilibrium, when the osmotic pressure of the interior equals that of the surrounding medium. This gain or loss of water is equal to, and may be directly measured by, the change in volume of the egg.

When in equilibrium with the external solution, we have already shown (8) that the product of pressure and volume of these cells is, as a first approximation, constant, and that

$$P_{\infty} V_{\infty} = P_0 V_0 \quad (1)$$

Here,  $P_{\infty}$  is the osmotic pressure of a solution with which the cell is in equilibrium, and therefore is also the pressure inside the egg;  $V_{\infty}$  is the cell volume at equilibrium;  $P_0$  and  $V_0$  are pressure and volume of the egg in ordinary sea water.

In the present paper we are dealing chiefly with cells not in equilibrium with the external solution and must therefore assume that the pressure-volume product is constant also under this condition. We write,

$$PV = P_0 V_0 \quad (1a)$$

where  $P$  and  $V$  are pressure and volume of the cell at any time.

When the egg is not in equilibrium with the external medium, we observe the process of swelling or of shrinking. According to assumption 5 the rate of transfer of water across unit area of membrane de-

depends only on the available pressure difference, and is proportional to it. Consequently the rate of transfer of water into or out of the egg will be proportional to the area of its surface, and to the difference in osmotic pressure between the interior and the external medium. This rate of transfer of water for the entire egg is measured directly by its rate of change of volume, and we write

$$\frac{dV}{dt} = k \cdot S \cdot (P - P_{ex}) \quad (\text{II})$$

Here  $\frac{dV}{dt}$  is the rate of change of volume of the egg;  $S$  is the area of the surface,  $P$  is the osmotic pressure of the interior of the egg,  $P_{ex}$  that of the surrounding medium,  $k$  is a factor of proportionality, which we shall term the *permeability of the cell to water*.\*

It is seen that the equation holds equally for swelling ( $\frac{dV}{dt} > 0$ ) or shrinking ( $\frac{dV}{dt} < 0$ ), and is so written that  $k$  is always a positive number. Moreover, it satisfies the condition for equilibrium ( $\frac{dV}{dt} = 0$ ), that the internal pressure equal that of the external medium.

Equation II cannot be integrated as it stands. However, Equation Ia furnishes the necessary relation between the internal osmotic pressure,  $P$ , and the volume of the egg.  $P_{ex}$  is a constant in the integration; for the sake of symmetry we shall, however, introduce the constant  $V_e$  given by Equation I.  $S$  is readily expressed in terms of  $V$ , and we have:

$$\frac{dV}{dt} = k (36 \pi)^{\frac{1}{3}} \cdot P_o V_o \cdot V^{\frac{2}{3}} \left( \frac{1}{V} - \frac{1}{V_e} \right) \quad (\text{II } a)$$

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\* This definition of permeability is seen to possess a definite physical meaning *viz.*, the number of cubic micra of water entering the cell per minute per unit area of membrane, per atmosphere of difference in osmotic pressure between interior and external medium. This definition of permeability has been used in our more recent papers (4, 5); its value may be computed directly from the graph of the swelling or shrinking processes.

In this differential equation the variables are the time,  $t$ , and the volume of the egg,  $V$ ; all other terms are constant. On integration we obtain the equation governing the expected course of swelling or shrinking:

$$k(36\pi)^{\frac{1}{2}} \cdot P_0 V_0 \cdot t = V_0 \left[ V_0^{\frac{1}{2}} \left( \frac{1}{2} \ln \frac{V_0^{\frac{1}{2}} + (V_0 V)^{\frac{1}{2}} + V^{\frac{1}{2}}}{(V_0^{\frac{1}{2}} - V^{\frac{1}{2}})^2} + \sqrt{3} \tan^{-1} \frac{2V^{\frac{1}{2}} + V_0^{\frac{1}{2}}}{\sqrt{3} V_0^{\frac{1}{2}}} \right) - 3V^{\frac{1}{2}} \right]_{V_0=0}^V \quad (\text{III})$$

where  $V$  is the volume of the eggs at time  $t$ . As indicated by the conventional notation, the term in the square brackets is to be taken between the limits  $V$  and  $V_0$ .\*

The treatment given by Northrop is somewhat different. He considers two cases: (a) the membrane contains pores whose diameter

\* We shall find it convenient subsequently to refer to the entire right hand side of this equation (without regard to limits) as  $f(V, V_0)$ , it being understood that in any one experiment of swelling or shrinking  $V_0$  is a constant, given by Equation I. We therefore write III simply

$$k(36\pi)^{\frac{1}{2}} \cdot P_0 V_0 \cdot t = f(V, V_0) \Big|_{V_0=0}^V \quad (\text{III } a)$$

For purposes of computation, it is helpful to note that  $f(V, V_0)$  is homogeneous in  $V$  and  $V_0$  and of degree  $\frac{3}{2}$ . We therefore have the identity

$$f(V, V_0) = (V_0)^{\frac{3}{2}} \cdot f\left(\frac{V}{V_0}, 1\right)$$

This has the distinct advantage that  $f(\frac{V}{V_0}, 1)$  may be computed once for all for a number of values  $\frac{V}{V_0}$ ; these values of  $f$  are then plotted against those of  $\frac{V}{V_0}$  as a conversion chart. The computation of  $f(V, V_0)$  is thus reduced to three steps:

1. Computation of  $\frac{V}{V_0}$ .
2. Finding, by means of the conversion chart,  $f(\frac{V}{V_0}, 1)$ .
3. Computation of  $f(V, V_0)$  by the identity given above. The construction of the conversion charts—one for swelling ( $\frac{V}{V_0} < 1$ ) and one for shrinking ( $\frac{V}{V_0} > 1$ )—forms the only laborious part of the work.

increases as the cell membrane is stretched in the swelling process; (b) the membrane, as it is stretched, does not alter in volume—its area increasing and its thickness decreasing. He then shows that both these assumptions lead to the same differential equation:

$$\frac{dV}{dt} = C_2 \cdot (36\pi)^{\frac{1}{2}} \cdot P_o V_o \cdot V^{\frac{1}{2}} \left( \frac{1}{V} - \frac{1}{V_e} \right) \quad \text{II (N)}$$

from which he writes

$$C_2 (36\pi)^{\frac{1}{2}} P_o V_o \cdot t = V_e^{\frac{1}{2}} \left[ \frac{1}{2} \ln \frac{V_e^{\frac{3}{2}} + (V_e V)^{\frac{1}{2}} + V^{\frac{1}{2}}}{(V_e^{\frac{1}{2}} - V^{\frac{1}{2}})^2} - \sqrt{3} \tan^{-1} \frac{2 V^{\frac{1}{2}} + V_e^{\frac{1}{2}}}{\sqrt{3} V_e^{\frac{1}{2}}} \right]_{V_t=0}^V \quad \text{III (N)}$$

where the notation is as in III.\* It is to be noted that Northrop's "C<sub>2</sub>" is different from the *k* of II and III.

#### EXPERIMENTS

The method employed in the present study is the same as has been previously described. Briefly stated, unfertilized eggs from a single specimen of *Arbacia punctulata* are placed in sea water which has been diluted with sufficient distilled water to give a solution having the desired osmotic pressure. Temperature control is accurate to  $\pm 0.5^\circ\text{C}$ . With an ocular micrometer and low power objective, the diameter of an egg is measured at minute intervals. Since the cells are spherical, volume can be calculated from diameter. Each volume recorded in this paper represents the mean value of six cells.

The first group of experiments here reported were designed to answer the question, Is the permeability of the cell affected by the salt concentration of the medium? Cells were taken from undiluted (100 per cent) sea water and placed in 20, 40, and 60 per cent sea water,

\* We have converted Northrop's notation to the form used in this paper.

We note once more that the right hand side is homogeneous in *V* and *V<sub>e</sub>*: we may, therefore, use the same method of computation by a conversion chart. Indeed, the presence of so many terms which are common to both Equations III and III<sub>N</sub> makes it possible to construct charts for both equations with very little additional labor.

respectively.\* The course of swelling was followed for a number of minutes. Data obtained from three experiments of this character are given in Table I. The same data have been used in Fig. 1, where

$$\frac{1}{(36\pi)^{\frac{1}{2}} \cdot P_0 V_0} \cdot f(V, V_0)$$

has been plotted against time. The resulting graphs are, in general, straight lines, showing that Equation III fits the data.\*\*

Moreover, these straight lines are approximately parallel, showing that there is no effect of salt concentration of the medium on permeability, at least under these conditions. Values of permeability factor  $k$  are given in Table I; these values show no drift with concentration.

In seven other experiments, in which various concentrations of sea water—20, 30, 40, 50, 60, 70 and 80 per cent—were used in different combinations, and at temperatures ranging from 15 to 21°C., the permeability factor was not found to vary in any definite way with the osmotic pressure of the medium.

Similar results are obtained by applying Northrop's equation. This is found to fit the data quite as well as Equation III. Constants calculated by means of Northrop's equation are given in Table I as values of  $C_2$ . These values also, it is seen, show no drift with change in concentration.

It is, therefore, concluded that under these conditions—that is, when cells previously in 100 per cent sea water are made to swell in various hypotonic dilutions of sea water, permeability as now defined is not affected by the osmotic pressure of the medium. The "velocity constant" of the unimolecular equation used in earlier papers has been shown to possess no physical significance, and the "concentra-

\* By 20 per cent sea water, for example, is meant a solution consisting of 20 parts of sea water and 80 parts of distilled water.

\*\* More accurately stated, the equation fits the data over the first few minutes of the experiment. The later points in the 20 per cent experiments are seen to rise above the straight line, indicating increase in permeability, which is probably due to injury, as cytolysis occurs a few minutes later. In contrast to this the later points in 60 per cent sea water (and especially in 70 and 80 per cent, as shown by unpublished experiments) tend to lie below the line, indicating that in these concentrations the equation does not fit the later part of the course of swelling.



tion effect" on this velocity constant is seen to be due to the empirical nature of the equation.

TABLE I

Data from three experiments (A, B, C) on the effect of salt concentration of the medium on permeability of the cell to water. The temperature was 15°C. The figures in the table must be multiplied by 100 to give the actual volumes in cubic micra. Each figure represents the mean volume of six cells. Volumes at times zero (0) have been obtained by extrapolation. In the bottom rows are given the values of the permeability factors obtained by the equation developed in this paper ( $k$ ), and by Northrop's equation ( $C_2$ ). The values of  $k$  indicate the number of cubic micra of water that pass through one square micron of cell surface per minute per atmosphere of pressure. The values of Northrop's constant are expressed in different units. It is seen that neither of these constants is affected by the salt concentration of the medium.

| Experiment:         | A      |        |        | B      |        |        | C      |        |        |
|---------------------|--------|--------|--------|--------|--------|--------|--------|--------|--------|
| Per cent sea water: | 20     | 40     | 60     | 20     | 40     | 60     | 20     | 40     | 60     |
| Time Min.           |        |        |        |        |        |        |        |        |        |
| 0                   | (2294) | (2294) | (2294) | (1960) | (2105) | (2045) | (2300) | (2300) | (2310) |
| 1                   | 2368   | 2377   | 2344   | 2135   | 2240   | 2139   | 2447   |        | 2405   |
| 2                   | 2543   | 2523   | 2447   | 2298   | 2358   | 2223   | 2666   | 2494   | 2494   |
| 3                   | 2716   | 2606   | 2523   | 2471   | 2494   | 2289   | 2819   | 2646   | 2548   |
| 4                   | 2850   | 2742   | 2582   | 2582   | 2591   | 2363   | 2914   | 2742   | 2616   |
| 5                   | 2967   | 2845   | 2646   | 2742   | 2666   | 2428   | 3092   | 2887   | 2656   |
| 6                   |        | 2914   | 2691   | 2903   | 2767   | 2480   | 3313   | 2962   | 2721   |
| 7                   | 3233   |        |        | 3092   |        |        | 3538   |        |        |
| 8                   | 3348   | 3114   | 2809   | 3279   | 2914   | 2557   | 3894   | 3114   | 2845   |
| 9                   | 3520   |        |        | 3556   |        |        |        |        |        |
| 10                  | 3629   | 3290   | 2898   |        | 3065   | 2646   |        | 3389   | 2940   |
| 11                  | 3798   |        |        |        |        |        |        |        |        |
| 12                  | 3965   | 3440   | 2962   |        | 3216   | 2731   |        | 3642   | 2994   |
| 14                  |        | 3580   | 3048   |        | 3348   | 2757   |        |        | 3059   |
| $k$                 | 0.051  | 0.052  | 0.051  | 0.058  | 0.055  | 0.055  | 0.050  | 0.049  | 0.050  |
| $C_2 \times 10^5$   | 2.50   | 2.56   | 2.55   | 3.13   | 2.79   | 2.94   | 2.62   | 2.58   | 2.60   |

We have furthermore noted that Northrop's treatment, based on different assumptions of membrane structure, is also satisfactory for describing the experimental data. Moreover, his differently defined "permeability,"  $C_2$ , is also seen to be independent of the concentra-

tion of the external medium. Indeed, the equation  $k = C_2S$ , which expresses the relation between Northrop's permeability and ours, assures us that, if the constants be determined (graphically) always for the same volume of the egg, they will be proportional to one another. We conclude that the small effect of concentration noted

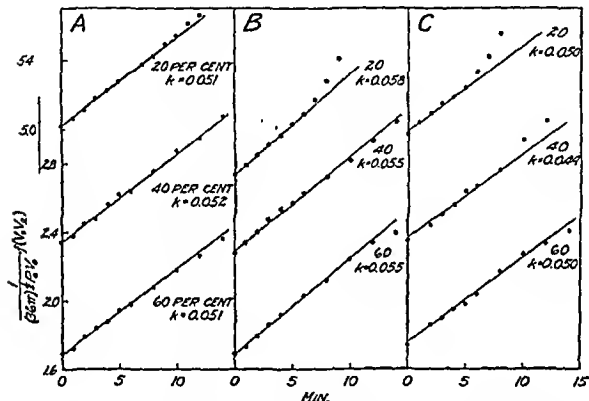


FIG. 1. The effect of salt concentration of the medium on the permeability of the cell to water. Data from three experiments (A, B, C) given in Table I, have been calculated by Equation III. When  $\frac{1}{(36\pi)^{\frac{1}{2}} P_0 V_0} \cdot f(V, V_0)$  is plotted against time the resulting graphs are, in general, approximately parallel straight lines, the slopes of which are the values of the permeability factor. It is seen that the equation fits the data over the first several minutes; the deviations at later minutes are discussed in the text.

by Northrop was due to the fact that the scanty data available to him at that time were less reliable than the present.

That the two equations should agree so closely is explained when a numerical comparison of the two is made. It is then seen that the difference between the two becomes appreciable only in extremes of concentration which cannot be employed with this material, or at

volumes so close to equilibrium that the experimental errors mask any real differences.\* In short, at the present time, it is impossible to choose between the two treatments on the basis of kinetics alone. In the absence of definite knowledge of membrane structure it is consequently a matter of indifference which is chosen.

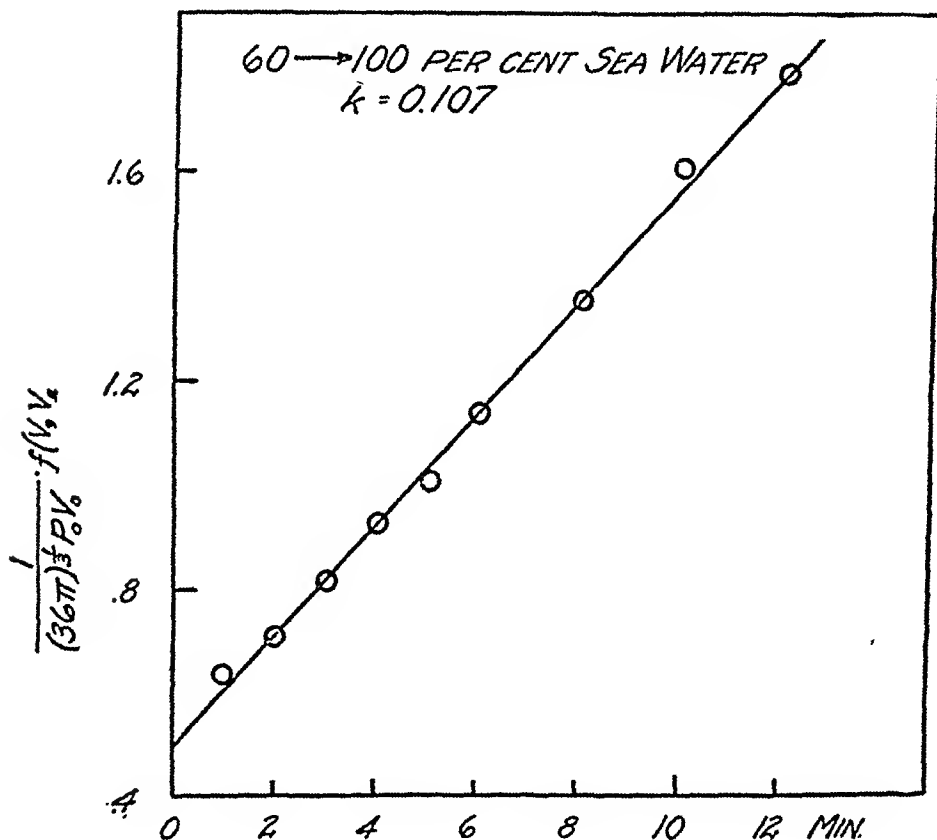


FIG. 2. The course of shrinking computed by Equation III. Cells previously swollen in 60 per cent sea water were returned to ordinary sea water in which they were measured during shrinking. The temperature was 15°C.

It is seen that a linear graph is obtained, showing that the equation fits the data.

As has been remarked, there is nothing in the derivation of the equation which in any way limits the treatment to swelling alone;

\* This is largely due to the presence, in both equations, of the term  $\ln (V_0^{\frac{1}{3}} - V^{\frac{1}{3}})$  which predominates over all the rest.

indeed it is found adequately to describe the shrinking process as well. This is shown to be the case in Fig. 2, which is typical of a large number of experiments. The cells, previously swollen in 60 per cent sea water, are returned to 100 per cent sea water and the course of shrinking measured. The plot of  $\frac{1}{(36\pi)^{\frac{1}{2}} P_o V_o} \cdot f(V, V_o)$  against time

is seen to be linear, indicating that the equation fits the data for shrinking. Northrop's equation describes the data equally well.

It is observed, however, that the value of  $k$  for shrinking is much greater than the values for swelling given in the preceding experiments. This difference has been found invariably in a large number of experiments in which swelling and shrinking have been compared.

A representative experiment is given in Table II. Cells have been measured while swelling in 60 per cent sea water at several temperatures. Other cells from the same animal, previously swollen in 60 per cent solution, have subsequently been measured while shrinking in ordinary sea water. Values of  $k$ , as determined by Equation III are given at the bottom of the table. It is seen that at corresponding temperatures, the value for shrinking is always greater than for swelling. The average ratio of shrinking to swelling in this experiment is 1.4.

Thus we reach the surprising conclusion that permeability, as defined by Equation III, is greater in shrinking than in swelling.

This at once suggests that Equation III may require modification. Indeed, one correction is already available. It was mentioned under assumption 1 that a fraction of cell volume is occupied by osmotically inactive material. Hence Equation Ia, heretofore used on account of its simplicity must be corrected for this fraction, which has been computed at 11 or 12 volume per cent (8). We now write for Ia

$$P(V - b) = P_o(V_o - b)$$

in which  $b$  is the volume occupied by osmotically inactive material.

Making the necessary substitutions in Equation IIa and integrating, we obtain

$$k(36\pi)^{\frac{1}{2}} \cdot P_o(V_o - b)t = (V_o - b) \left[ \left(1 - \frac{b}{V_o}\right) \cdot V_o^{\frac{1}{2}} \left( \frac{1}{2} \ln \frac{V_o^{\frac{1}{2}} + (V_o V)^{\frac{1}{2}} + V^{\frac{1}{2}}}{(V_o^{\frac{1}{2}} - V^{\frac{1}{2}})^{\frac{1}{2}}} \right. \right. \\ \left. \left. + \sqrt{3} \tan^{-1} \frac{2V^{\frac{1}{2}} + V_o^{\frac{1}{2}}}{\sqrt{3} V_o^{\frac{1}{2}}} \right) - 3V^{\frac{1}{2}} \right]_{V_t=0}^V \quad (\text{IV})$$

TABLE II

Permeability to water during swelling compared with that during shrinking at several temperatures. The upper half of the table represents the course of swelling in 60 per cent sea water, the lower half, the course of shrinking of cells returned from 60 per cent sea water to ordinary sea water. Permeability has been computed by Equation III ( $k_3$ ) and by Equation IV ( $k_4$ ),  $b$  has been taken as 12 per cent of the volume of cells in ordinary sea water. It is seen that there is less difference in the values of  $k_4$  in swelling and shrinking than in the values of  $k_3$ .

Each figure represents the mean volume of six cells. The figures must be multiplied by 100 to obtain volumes in cubic micra. The mean volume of 20 control cells in ordinary sea water was  $2033 \times 10^2 \mu^3$ .

|                     | Time        | 12°   | 15°   | 18°   | 21°   | 24°   |
|---------------------|-------------|-------|-------|-------|-------|-------|
|                     | <i>min.</i> |       |       |       |       |       |
| Course of swelling  | 1           | 2276  | 2187  | 2317  | 2331  | 2313  |
|                     | 2           | 2368  | 2253  | 2428  | 2447  | 2471  |
|                     | 3           | 2400  | 2322  | 2504  | 2557  | 2577  |
|                     | 4           | 2447  | 2381  | 2548  | 2631  | 2691  |
|                     | 5           | 2485  | 2437  | 2606  | 2696  | 2778  |
|                     | 6           | 2509  | 2494  | 2651  | 2731  | 2834  |
|                     | 8           | 2596  | 2572  | 2757  | 2819  | 2903  |
|                     | 10          | 2646  | 2681  | 2834  | 2850  | 2978  |
|                     | 12          | 2716  | 2721  | 2887  | 2914  |       |
|                     | 14          | 2783  | 2778  | 2967  | 2967  |       |
|                     | 16          | 2834  | 2809  | 3016  | 3000  |       |
|                     | 18          | 2850  | 2809  | 3026  | 3037  |       |
|                     | 20          | 2898  | 2876  | 3070  | 3081  |       |
|                     | 22          | 2919  | 2898  |       |       |       |
|                     | 24          | 2940  |       |       |       |       |
|                     | $k_3$       | 0.043 | 0.051 | 0.071 | 0.107 | 0.135 |
|                     | $k_4$       | 0.047 | 0.059 | 0.079 | 0.111 | 0.156 |
| Course of shrinking | 1           | 2871  | 2819  | 2861  | 2757  | 2606  |
|                     | 2           | 2772  | 2716  | 2778  | 2596  | 2428  |
|                     | 3           | 2691  | 2666  | 2606  | 2485  | 2344  |
|                     | 4           | 2631  | 2562  | 2523  | 2381  | 2209  |
|                     | 5           | 2562  | 2494  | 2461  | 2326  | 2179  |
|                     | 6           | 2499  | 2447  | 2400  | 2244  | 2152  |
|                     | 8           | 2442  | 2353  | 2313  | 2165  | 2101  |
|                     | 10          | 2395  | 2285  | 2262  | 2109  | 2092  |
|                     | 12          | 2335  | 2227  | 2240  | 2092  | 2079  |
|                     | 14          | 2335  | 2218  | 2205  |       |       |
|                     | 16          | 2298  |       |       |       |       |
|                     | $k_3$       | 0.067 | 0.074 | 0.098 | 0.142 | 0.175 |
|                     | $k_4$       | 0.059 | 0.067 | 0.086 | 0.128 | 0.157 |

Equation IV has been found to fit the data, if anything, a little better than Equation III.\* Applying it to the data of Table II, we obtain the values for permeability given in the bottom row. It is seen that  $k$  for swelling is slightly increased while  $k$  for shrinking is distinctly reduced. The average ratio of shrinking to swelling now becomes slightly greater than 1.1.\*\*

TABLE III

Permeability to water during swelling in 60 per cent sea water compared with that during shrinking in cells returned from 60 per cent sea water to ordinary sea water. Permeability has been computed in several experiments by Equation III ( $k_3$ ) and Equation IV ( $k_4$ ).  $b$  has been taken as 12 per cent of the volume of cells in ordinary sea water. The mean ratio of shrinking to swelling is, for  $k_3$ , 1.51; for  $k_4$ , 1.25.

| Temp. | Swelling | Shrinking | Ratio | Swelling | Shrinking | Ratio |
|-------|----------|-----------|-------|----------|-----------|-------|
| *C.   | $k_3$    | $k_3$     |       | $k_4$    | $k_4$     |       |
| 20    | 0.081    | 0.155     | 1.91  | 0.087    | 0.141     | 1.62  |
| 12    | 0.043    | 0.067     | 1.56  | 0.047    | 0.059     | 1.26  |
| 15    | 0.051    | 0.074     | 1.45  | 0.059    | 0.067     | 1.14  |
| 18    | 0.071    | 0.098     | 1.38  | 0.079    | 0.086     | 1.09  |
| 21    | 0.107    | 0.142     | 1.33  | 0.111    | 0.128     | 1.15  |
| 24    | 0.135    | 0.175     | 1.30  | 0.156    | 0.157     | 1.01  |
| 12    | 0.041    | 0.090     | 2.19  | 0.045    | 0.076     | 1.69  |
| 15    | 0.051    | 0.086     | 1.69  | 0.057    | 0.076     | 1.33  |
| 18    | 0.079    | 0.119     | 1.51  | 0.083    | 0.106     | 1.28  |
| 21    | 0.098    | 0.125     | 1.28  | 0.104    | 0.106     | 1.02  |
| 24    | 0.133    | 0.200     | 1.50  | 0.136    | 0.184     | 1.35  |
| 18    | 0.085    | 0.118     | 1.39  | 0.084    | 0.105     | 1.24  |
| 18    | 0.077    | 0.091     | 1.18  | 0.081    | 0.082     | 1.01  |

However, this ratio varies considerably in different experiments. In Table III, values of  $k_3$  and  $k_4$  are given for a number of experiments,

\* A similar correction can of course be applied to Northrop's equation.

\*\* While Equation IV describes the course of osmosis more accurately, it is relatively unwieldy. Therefore Equations II and III are to be preferred when no serious error is introduced by their use; this is the case in experiments in which cells from ordinary sea water are swelled in hypotonic dilutions.

together with the ratios of these permeability factors for shrinking and swelling. The mean ratio of shrinking to swelling when computed by Equation III is 1.51; by Equation IV is 1.25.

Thus Equation IV reduces the difference between shrinking and swelling by one-half, on the average. There still remains a significant difference between the two processes; water leaves the cell more readily than it enters. Consideration of the forces, such as elasticity and surface tension, and of mechanisms within the cell which might bring about this result will be made the subject of a future paper.

#### DISCUSSION

In the present paper a theoretical treatment of osmosis has been developed which we believe marks a step in advance. Permeability has been defined in terms having definite physical meaning. So defined, permeability is found no longer to be dependent on the osmotic pressure of the medium, and so we have simplified our conception of the mechanism by which the flow of water across the cell surface is regulated. Our working hypothesis in its present form has already been found useful in measuring changes in permeability brought about by various environmental factors. That the hypothesis is subject to further modification is suggested by the observations that permeability, as we have defined it, is not constant after the first few minutes of the swelling and shrinking processes, and that its numerical value is greater in shrinking than in swelling.

#### SUMMARY

Using unfertilized eggs of *Arbacia punctulata* as natural osmometers an attempt has been made to account for the course of swelling and shrinking of these cells in anisotonic solutions by means of the laws governing osmosis and diffusion. The method employed has been to compute permeability of the cell to water, as measured by the rate of volume change per unit of cell surface per unit of osmotic pressure outstanding between the cell and its medium.

Permeability to water as here defined and as somewhat differently defined by Northrop is approximately constant during swelling and shrinking, at least for the first several minutes of these processes.

Permeability is found to be independent of the osmotic pressure of the solution in which cells are swelling.

Water is found to leave cells more readily than it enters, that is, permeability is greater during exosmosis than during endosmosis.

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# ON THE TEMPERATURE CHARACTERISTICS FOR FREQUENCY OF BREATHING MOVEMENTS IN INBRED STRAINS OF MICE AND IN THEIR HYBRID OFFSPRING. I

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## I

In conjunction with a series of investigations concerned with hereditary behavior of the geotropic response of young rats (Crozier and Pincus, 1928-29; Pincus and Crozier, 1929), an attempt was made to ascertain whether similar relations could be discovered for an entirely different kind of activity, namely, the temperature relations of the frequency of breathing movements of young mice. An initial investigation established the fact that the data relating frequency of breathing movements to temperature could be described with sufficient accuracy by the Arrhenius equation (Stier and Pincus, 1928). Using essentially the same technique, but with improved methods of temperature regulation,<sup>1</sup> the present investigation was begun with a study of the long-inbred strain of *dilute brown* mice. These were mice obtained from Dr. C. C. Little, which had undergone strict brother-sister mating for twelve generations and previous to this had been inbred for some years. The animals used in these experiments were of the sixteenth to nineteenth generation of strict brother-sister mating.

\* National Research Council Fellow (1927-30).

<sup>1</sup>The mice were placed in an air chamber immersed in a thermostat of the type described by Crozier and Stier (1927). After any setting the temperature of the chamber never varied by more than 0.1°C., usually by much less. Readings were made of the time taken for either five or ten respiratory beats, and from twenty to sixty single observations were made at each temperature. All readings were made when the animal lay perfectly quiet, and *none* were taken during periods of movement. For other details see Stier and Pincus (1928).



# ON THE TEMPERATURE CHARACTERISTICS FOR FREQUENCY OF BREATHING MOVEMENTS IN INBRED STRAINS OF MICE AND IN THEIR HYBRID OFFSPRING. I

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## I

In conjunction with a series of investigations concerned with hereditary behavior of the geotropic response of young rats (Crozier and Pincus, 1928-29; Pincus and Crozier, 1929), an attempt was made to ascertain whether similar relations could be discovered for an entirely different kind of activity, namely, the temperature relations of the frequency of breathing movements of young mice. An initial investigation established the fact that the data relating frequency of breathing movements to temperature could be described with sufficient accuracy by the Arrhenius equation (Stier and Pincus, 1928). Using essentially the same technique, but with improved methods of temperature regulation,<sup>1</sup> the present investigation was begun with a study of the long-inbred strain of *dilute brown* mice. These were mice obtained from Dr. C. C. Little, which had undergone strict brother-sister mating for twelve generations and previous to this had been inbred for some years. The animals used in these experiments were of the sixteenth to nineteenth generation of strict brother-sister mating.

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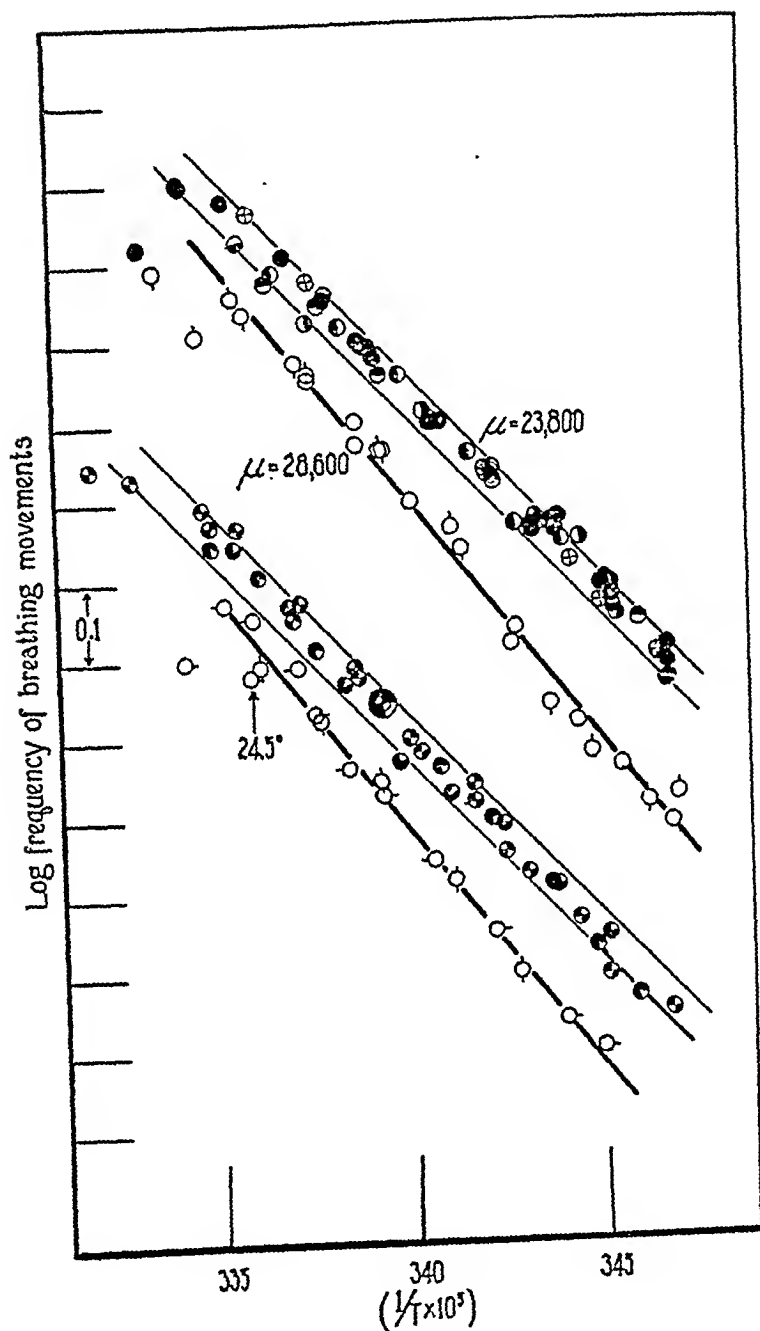


FIG. 1. Upper curves: Mass plots of data from individual mice of a selected line of the *dilute brown* strain. The data from six individuals (closed or partly closed circlets) give an average value of 23,800 calories for the temperature char-

In collaboration with Dr. T. J. B. Stier the writer undertook a preliminary survey of this strain, using unselected individuals from various lines within the strain. A summary of the results is given in Table I. It will be noted that five distinct modal values of the critical increment were obtained. In association with those most frequently observed, namely  $\mu = 20,000 \pm$  and  $\mu = 28,000 \pm$ , we observed invariably a critical temperature in the region of  $25^{\circ}\text{C}$ . (usually at about  $24.5^{\circ}\text{C}$ .). From  $15$ – $25^{\circ}\text{C}$ . there was a regular increase of the frequency of breathing movements; beyond  $25^{\circ}\text{C}$ . there was either no change in the frequency (*i.e.*, a constant rate was maintained), or there was a sudden decrease followed by a regular increase so that from  $25$ – $35^{\circ}\text{C}$ . there was reproduced the value of  $\mu$  obtained over the range  $15$ – $25^{\circ}\text{C}$ .

I then selected for final test only the offspring of a given male by his sisters. Out of nine young, six gave the value  $\mu = 24,000 \pm$  (average =  $23,800$ ) over the range  $15$ – $25^{\circ}\text{C}$ ., and three gave the value  $\mu = 28,000 \pm$  (average =  $28,600$ ) over the range  $15$ – $25^{\circ}\text{C}$ . (see Fig. 1). This male, his sisters, and their offspring were used exclusively in the succeeding crosses. Dilute brown animals No. 1 to 6 were tested in October, 1928, and Animals No. 7 to 12 were tested in April, 1929, the latter representing sibs from the second generation of the original male (No. 10,011). The animals tested were from 2 to 6 days

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acteristic. The data from three individuals (open circles) give an average value of  $28,600$  calories for the temperature characteristic.

Lower curves: Mass plots of data from individual mice of the backcross generation  $F_1$  by *dilute brown* (over the range  $15$ – $25^{\circ}\text{C}$ .). Four individuals (closed or partly closed circles) showed a value of  $24,000 \pm$  calories for the temperature characteristic, while three showed a value of  $28,000 \pm$ .

The lines drawn through the data for the backcross generation are exactly parallel to those drawn for the parental strain data. The data are brought together in this and in succeeding mass plot figures by multiplication of the various sets by respective appropriate constants. Data of this and all succeeding figures were obtained in some cases by beginning observations at a low temperature and proceeding up the temperature scale, or by reversing this procedure, or by alternating "high" and "low" temperatures. Each plotted point represents an average of twenty to fifty single observations, generally thirty-five. There has been discovered no relation between the direction in which temperature changes were made and the resultant values of the derived constants.

old. This represents the range of ages ordinarily used in the succeeding experiments. These data, and those of Tables I and II, demonstrate that there is no significant change of the value of the constant  $\mu$  within the range of ages and of weights employed. Apparently, however, within the pure line alternative values of the constant, namely,  $\mu = 24,000 \pm$  or  $\mu = 28,000 \pm$ , may be exhibited. Further-

TABLE I

*Data from Unselected Individuals from Several Lines of the Dilute Brown Strain of Mice*

| No. of instances | Age of animal | Weight     | Modal $\mu$ | Extreme range |
|------------------|---------------|------------|-------------|---------------|
|                  | <i>days</i>   | <i>gm.</i> |             |               |
| 1                | 6             | 1.94       | 14,000      | 14,080        |
| 3                | 2             | 1.72       | 16,000      | 15,950-16,570 |
|                  | 5             | 2.23       |             |               |
|                  | 3             | 2.14       |             |               |
| 6                | 3             | 2.50       | 20,000      | 19,709-20,800 |
|                  | 6             | 3.59       |             |               |
|                  | 2             | 1.26       |             |               |
|                  | 4             | 1.76       |             |               |
|                  | 7.5           | 3.98       |             |               |
|                  | 10            | 3.68       |             |               |
| 6                | 1             | 1.71       | 28,000      | 27,500-28,460 |
|                  | 10            | 3.13       |             |               |
|                  | 8             | 2.80       |             |               |
|                  | 12            | 3.31       |             |               |
|                  | 3             | 2.14       |             |               |
|                  | 5.5           | 3.14       |             |               |
| 1                | 10.5          | 6.31       | 34,000      | 34,215        |

more, a single individual may on different occasions exhibit either of these alternative values. As an example an individual of the first series (Table I) gave the value  $\mu = 28,200$  over the range 15-25°C. when it was 2 days old, the value  $\mu = 20,100$  when it was 4 days old, and again the value  $\mu = 28,200$  when it was 6 days old.

A second strain of inbred mice (the *Bagg albinos*) was obtained from Dr. L. C. Strong. A preliminary examination of seven individuals of

the twenty-eighth brother-sister generation showed that an entirely different situation existed in this strain. All the individuals examined exhibited a definite critical temperature in the region of 20°C. (usually at about 19.4°C.). The value of the constant  $\mu$  over the range 15-

TABLE II

Summary of data relating frequency of breathing movements to temperature in animals of the inbred albino strain (twenty-eighth to thirtieth brother-sister generation).

| Animal no. | Value of $\mu$ |             | Age         | Weight     |
|------------|----------------|-------------|-------------|------------|
|            | 15 to 20°C.    | Above 20°C. |             |            |
|            |                |             | <i>days</i> | <i>gm.</i> |
| 2 L6       |                | 14,600      | 7           | 2.80       |
| 1 L5       | 32,800         |             | 4           | 1.95       |
| 2 L7       |                | 13,900      | 6           | 2.60       |
| 1 L3       |                | 14,000      | 7           | 2.20       |
| 1 L3       | 34,600         | 14,000      | 3           | 1.30       |
| 1 L4       |                | 14,100      | 2           | 1.40       |
| 2 L6       | 32,700         |             | 7           | 2.69       |
| 2 L4(a)    | 33,000         | 14,200      | 6           | 2.15       |
| 2 L4(b)    | 34,500         | 14,400      | 9           | 3.25       |
| 1 L7(a)    |                | 13,700      | 4           | 1.81 (1)   |
| 1 L6       |                | 13,800      | 6           |            |
| 3 L7       | 14,300         | 14,100      | 6           | (2)        |
| 1 L7(b)    | 34,400         | 14,200      | 8           | (3)        |
| A          | 14,800         | 13,900      | 6           | 2.50       |
| C(a)       | 14,600         | 14,500      | 3           |            |
| B          | 33,900         |             |             | 3.70       |
| C(b)       | 32,800         | 14,800      | 5           |            |

(1) Nursed by dbr.

(2) Nursed by dbr.

(3) Nursed by dbr.

(a) First trial.

(b) Second trial.

20°C. was usually  $34,000 \pm$ , but in a few cases the value was  $14,000 \pm$ . From 20°C. upward the value obtained was always  $14,000 \pm$  (see Table II). The critical temperature at 20°C. was demonstrated by either a change of increment from  $34,000 \pm$  to  $14,000 \pm$  or by an abrupt shift in the frequency of breathing movements (see Figs. 2 and 3).



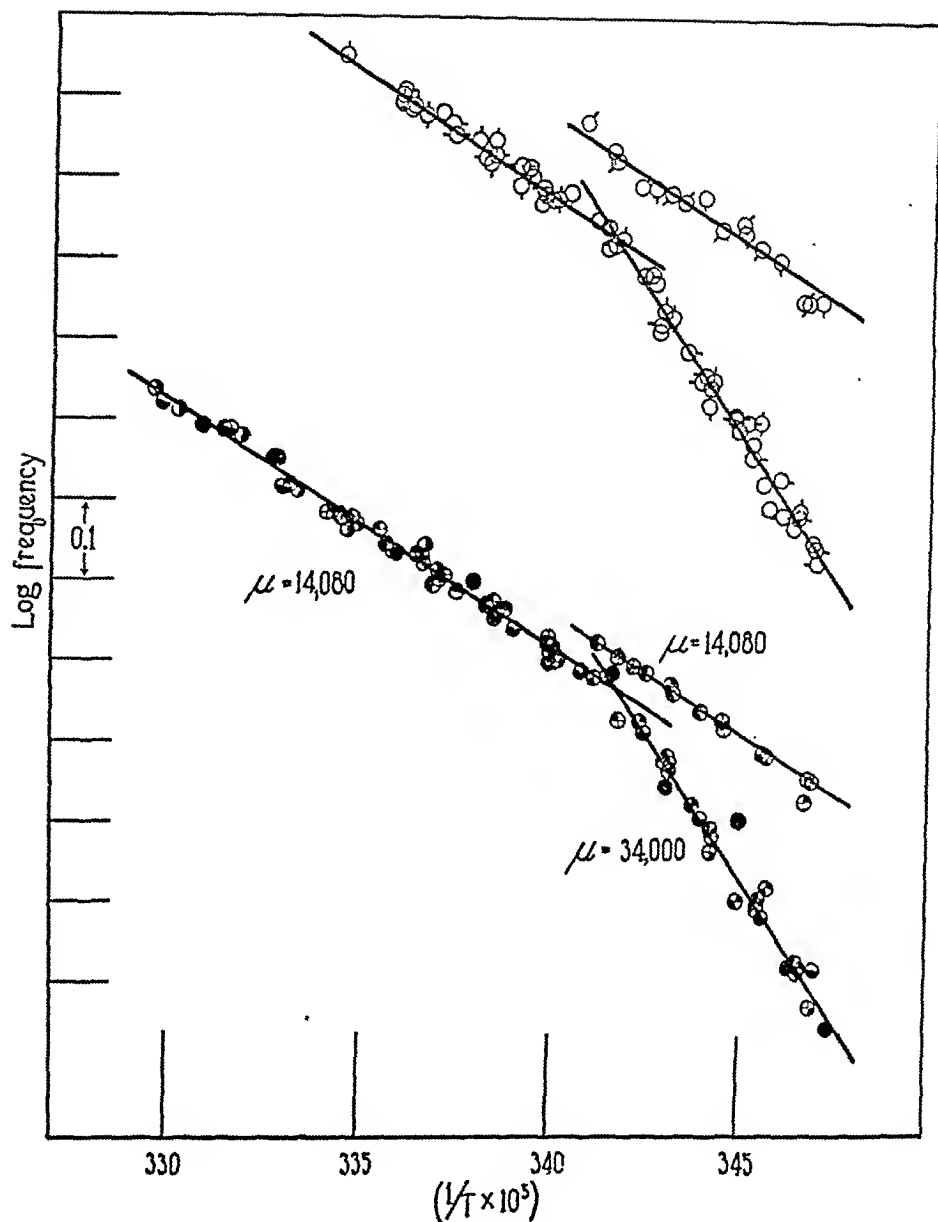


FIG. 2. Lower curves: Mass plot of data from eleven individuals (closed or partly closed circles) of an inbred *albino* strain. Data from these individuals always give evidence of a critical temperature in the region of 20°C., ordinarily by a change in value of the temperature characteristic such that above 20°C. the value is  $14,000 \pm$  calories, while from 15–20°C. this value is  $34,000 \pm$  calories, or by an abrupt shift in the frequency of breathing movements at about 20°C. and a value of  $14,000 \pm$  calories from 15–20°C. Thus in all cases the value of the temperature characteristic is  $14,000 \pm$  calories above 20°C., and it may be either  $34,000 \pm$  calories or  $14,000 \pm$  calories from 15 to 20°C.

Upper curves: Mass plot of data from nine individuals of the backcross generation  $F_1$  by *albino*. All the data plotted give a value of  $14,000 \pm$  calories for the temperature characteristic from 20°C. upward; five individuals give  $34,000 \pm$  from 15–20°C.; three individuals give  $14,000 \pm$  from 15–20°C.

The preliminary findings were substantiated by later repetitions with the offspring of a single male by his sisters. Again, only the members of this selected line were used in subsequent crosses, although all the albinos exhibited essentially the relationship described.

## II

It should be pointed out that the data from all these experiments are plotted against the internal temperature of the animals as recorded by a rectal thermopile. Justification for using internal rather than external temperature is had not merely in the logical consideration that the reactions involved are going on in the animal and therefore at the temperature of the body itself, but also in the obvious consistency of the data when the internal temperature is used. This is best illustrated by Figs. 3 and 4. Fig. 3 shows data for an animal (No. 214) of the albino strain which were obtained on two different days; the upper curve represents data obtained when the animal was 9 days old; the data for the lower curve were taken when the animal was 6 days old. The lines fitted to the data are obviously parallel, but the two sections are not equidistant, due to the occurrence of the critical point at slightly different temperatures ( $0.7^{\circ}$  apart) in the two cases. When, however, as in Fig. 4, the data are plotted against external temperature rather than against body temperature, there is no parallelism of the curves; and while an attempt may be made to fit the data by the Arrhenius equation this would not only be meaningless but the fit is not good, and the *apparent* values of the constant  $\mu$  obtained are really dependent on the temperature of the animal's body.

It has been pointed out previously (Stier and Pincus, 1928) that the internal temperature of young mice, within certain temperature limits, is never that of the environment, but always somewhat higher. A young mouse placed in a chamber where the external temperature is different from the body temperature becomes cooled or warmed in a regular manner, until the internal temperature reaches a certain fairly constant level. The curve of Fig. 5 is a typical cooling curve showing the change in internal temperature after a constant external temperature is attained. It will be noted that the curve is asymptotic. Analysis of such curves is reserved for future consideration. It is

sufficient to note here that readings of the frequency of respiratory movements were taken at the approximately constant level of internal

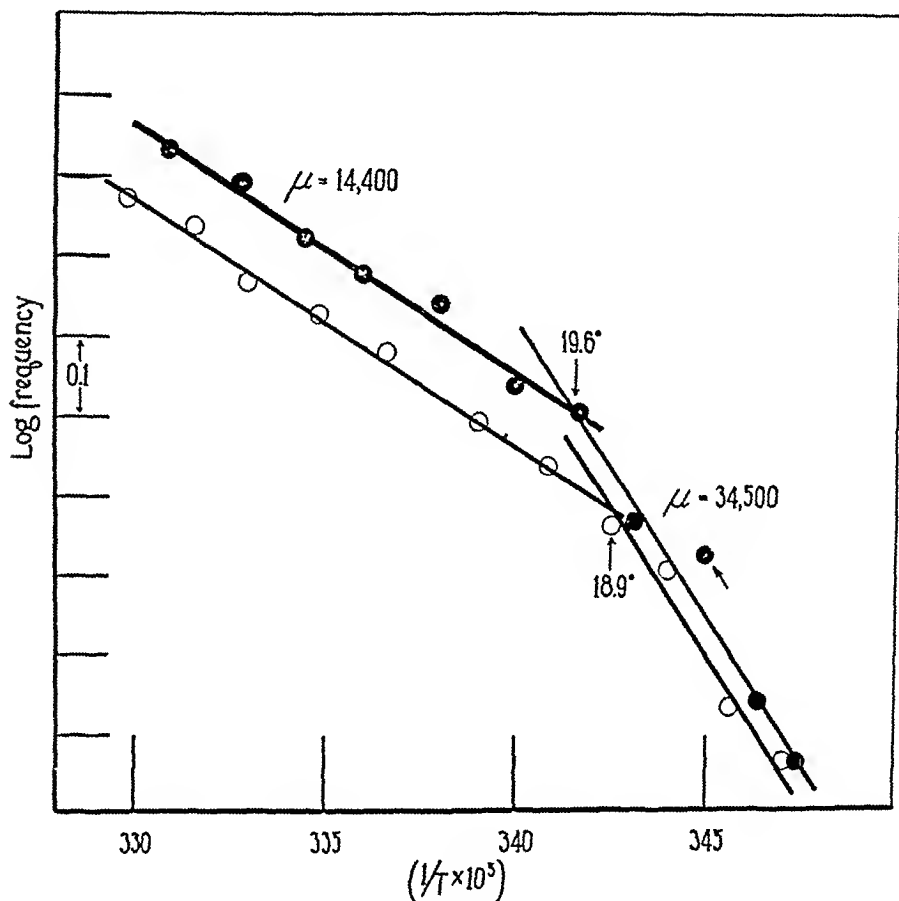


FIG. 3. Data for inbred *albino* animal No. 2L4, taken on two successive occasions. The open circles represent data taken when the mouse was six days old, the closed circles data taken when the mouse was nine days old. These data (as all the data of these experiments) are plotted against the *internal* temperature of the animal. Note an increase in the absolute rate of respiratory movements when the mouse is older, but no change in the value of the slope constants. See text and Fig. 4. The one point at  $16.82^\circ$  which deviates markedly from the line drawn through the data is derived from observations which show a comparatively high probable error (the coefficient of variation is about twice that of the points on either side); none the less these data do contain a number of single readings which fall on the line drawn.

temperature represented by the extreme lowermost portion of the curve (Fig. 5), with simultaneous and continuous recording of the

internal temperature. It is to be noted further that the residual difference between the internal and external temperatures is itself a function of temperature, the difference increasing with increasing internal temperature.

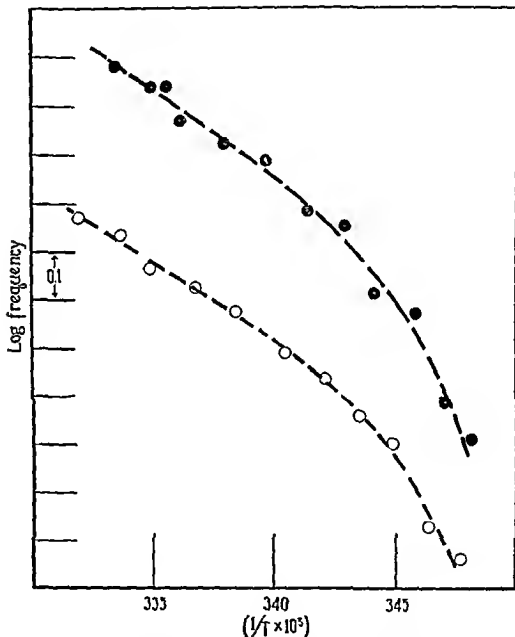


FIG. 4. The breathing frequencies of Fig. 3, plotted against the external (air) temperature. The curves are in no sense parallel. See text.

In the  $F_1$  and backcross generations to be described, experiments were chiefly carried on over the range 15–25°C., in order to obtain accurate tests over a limited range and because of the complicating occurrence of a critical temperature at 25°C. in many cases.

In the two inbred lines some of the animals used were put to nurse with mothers of the other inbred line (*cf.* Table II), to ascertain whether the value of the temperature characteristic might be modified through the mother. In every case the temperature characteristic obtained was that typical for the young mouse's strain, never that of the nurse mother's strain, although the animals tested were nursed

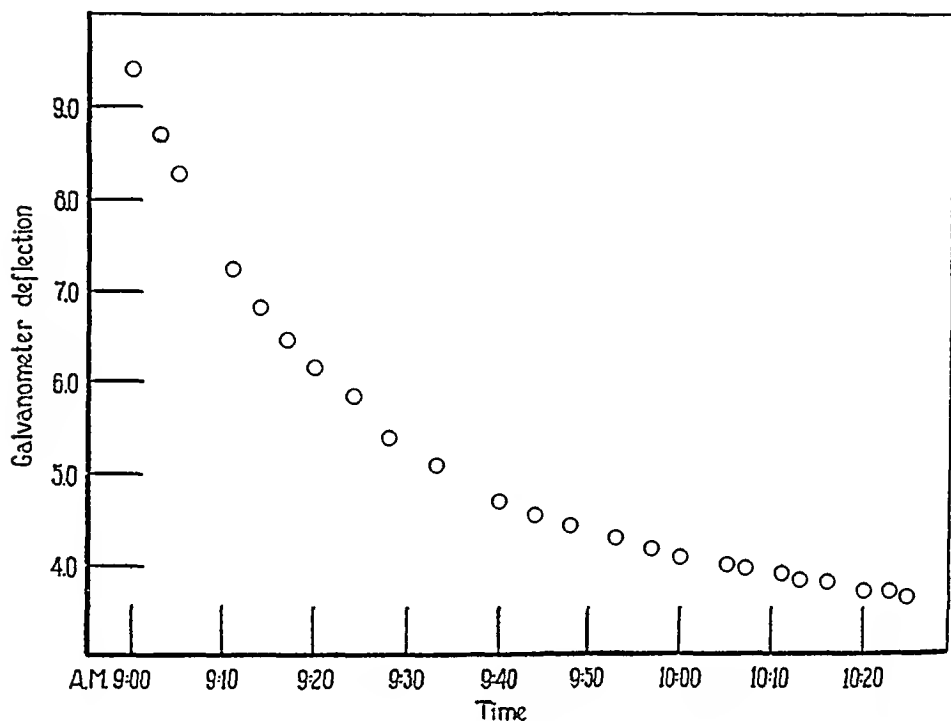


FIG. 5. Curve showing the rate of cooling of a young mouse after the external (air) temperature had become relatively constant ( $14.87$ – $14.83^{\circ}\text{C}.$ ). The galvanometer deflection is a measure of the difference between the rectal temperature and the external temperature. Each division of the galvanometer scale equals  $0.294^{\circ}\text{C}.$

for as long as 9 days by the foster mother. In the  $F_1$  generation four young were nursed by *dilute brown* mothers, and eight by *albino* mothers (Table III). Again there was no apparent correlation between the temperature characteristics for the young and the type of nurse mother.

## III

Table III gives the results obtained on testing twelve  $F_1$  animals from reciprocal crosses of the two selected lines of animals. In all these except one, a definite critical temperature occurred at  $19.5 \pm 0.7^\circ\text{C}$ ., as in the albino strain. It will be seen that in general the value of the temperature characteristic exhibited over the range  $15\text{--}20^\circ\text{C}$ . is  $24,000 \pm$ , while beyond  $20^\circ\text{C}$ . the value most frequently obtained is  $14,000 \pm$ . Thus over the range  $15\text{--}20^\circ\text{C}$ . the values typical for the *dilute brown* strain are exhibited, while beyond  $20^\circ\text{C}$ . the value typical for the *albino* strain is had. There are exceptions to

TABLE III  
*F<sub>1</sub>-Dilute Brown*  $\times$  *Albino*

| Animal no. | Value of $\mu$             |                            | Mother              |
|------------|----------------------------|----------------------------|---------------------|
|            | 15 to $20^\circ\text{C}$ . | Above $20^\circ\text{C}$ . |                     |
| $F_1$ 1    | 28,380                     | 14,280                     | <i>dilute brown</i> |
| 2          | 24,600                     | 14,600                     | "                   |
| 3          | 23,640                     |                            | "                   |
| 4          | 24,370                     | 14,280                     | <i>albino</i>       |
| 5          | 24,700                     | 14,600                     | "                   |
| 7          | 23,700                     | 14,200                     | "                   |
| 8          | 34,800                     | 14,230                     | "                   |
| 9          | 24,100                     |                            | "                   |
| 11         | 23,400                     | 14,600                     | "                   |
| 12         | 13,800                     | 24,300                     | <i>dilute brown</i> |
| 13         | 23,700                     | 14,600                     | <i>albino</i>       |
| 14         | 24,150                     | 24,150*                    | "                   |

\* No break at  $20^\circ\text{C}$ .

this (Table III), but in no case is any value obtained which has not been observed in one or the other of the two parent strains. What one might expect in the progeny of such a cross is *a priori* unpredictable. The ordinary Mendelian conception of dominance and recessiveness is not necessarily applicable here, because we are dealing with a two-coordinate system, whereas the genic effect ordinarily studied represents the end-point of a process, not the process itself (*cf.* Crozier and Pincus, 1929). Were it not for the exceptional occurrence of  $\mu = 24,000 \pm$  from  $20^\circ\text{C}$ . upward and of 34,000 or of 14,000 from  $15\text{--}20^\circ\text{C}$ ., it would

obtained in the two backcrosses as well as certain types of curves not found in the parent strains or generally in the  $F_1$  generation. The upper and lower curves illustrate the reality of the "shift" at 20°C. The middle curve gives the rather unusual situation in which the value of  $\mu$  is less at the lower temperatures than at the upper tempera-

TABLE V  
 $F_1 \times \text{Albino}$

| Animal no. | Value of $\mu$ |             |
|------------|----------------|-------------|
|            | 15 to 20°C.    | Above 20°C. |
| 1          | 24,600         | 14,600      |
| 2          | 24,200         | 13,700      |
| 6          | 23,500         | 14,300      |
| 14         | 24,600         | 13,500      |
| 17         | 23,800         | 14,400      |
| 28         | 24,000         | 14,300      |
| 29         | 23,900         | 14,700      |
| 41         | 24,300         | 15,100      |
| 45         | 24,200         | 13,500      |
| 3          | 14,200         | 13,600 (?)  |
| 5          | 14,600         | 14,900      |
| 40         | 14,600         | 14,400      |
| 4          | 32,500         | 14,000      |
| 8          | 34,800         | 14,400      |
| 11         | 33,000         | 14,100      |
| 15         | 34,600         | 13,700      |
| 39         | 33,800         |             |
| 44         | 34,600         | 24,200      |
| 16         | 28,000         |             |
| 42         | 28,300         | 14,600      |
| 12         | 14,300         | 23,800      |
| 7          | 23,700         | 23,900      |
| 27         | 24,000         | 23,600      |
| 42         | 24,300         | 28,500      |

tures. Such a combination ( $\mu = 14,000 \pm$  from 15–20°C. and  $24,000 \pm$  from 20°C. upward) is, however, to be expected if there exists a genetic recombination of the various types of curves observed in the parent strain. It will be noted again that the values of  $\mu$  are *only* those values observed in the parent strains, though many other values "might" have been obtained (*cf.* Stier and Pincus, 1928, *and* Table I).

Fig. 8 assembles three individual curves out of the backcross. The upper and middle curves are from data on the same indi

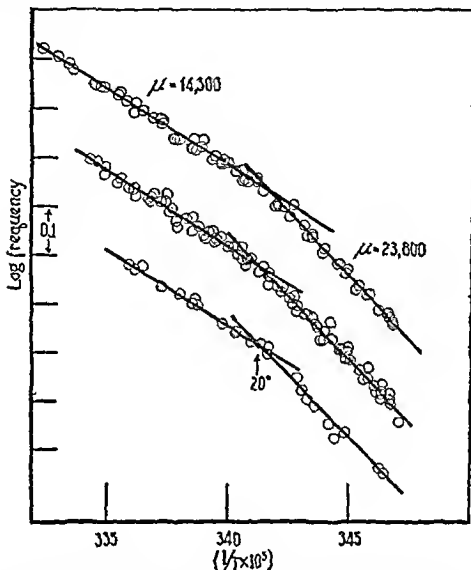


FIG. 6. Upper curve: Data from five individuals of the  $F_1$  generation gave the same values of the temperature characteristic, namely,  $24,000 \pm 15$ – $20^\circ\text{C}$ . and  $14,000 \pm$  from  $20^\circ\text{C}$ . upwards. Middle curve: Data from individuals of the backcross generation  $F_1 \times \text{albino}$ .

Lower curve: data from three individuals of the backcross generation *dilute brown*. The lines drawn through the data for the two backcross generations are exactly parallel to those drawn through the  $F_1$  data. (No attempt made to distinguish individuals by separate symbols.)

taken on successive occasions. The existence of a critical temperature at  $20^\circ\text{C}$ . is demonstrable in one instance (middle curve) by an abrupt shift in the rate as well as by a change in the value of the



obtained in the two backcrosses as well as certain types of curves not found in the parent strains or generally in the  $F_1$  generation. The upper and lower curves illustrate the reality of the "shift" at 20°C. The middle curve gives the rather unusual situation in which the value of  $\mu$  is less at the lower temperatures than at the upper tempera-

TABLE V  
 $F_1 \times \text{Albino}$

| Animal no. | Value of $\mu$ |             |
|------------|----------------|-------------|
|            | 15 to 20°C.    | Above 20°C. |
| 1          | 24,600         | 14,600      |
| 2          | 24,200         | 13,700      |
| 6          | 23,500         | 14,300      |
| 14         | 24,600         | 13,500      |
| 17         | 23,800         | 14,400      |
| 28         | 24,000         | 14,300      |
| 29         | 23,900         | 14,700      |
| 41         | 24,300         | 15,100      |
| 45         | 24,200         | 13,500      |
| 3          | 14,200         | 13,600 (?)  |
| 5          | 14,600         | 14,900      |
| 40         | 14,600         | 14,400      |
| 4          | 32,500         | 14,000      |
| 8          | 34,800         | 14,400      |
| 11         | 33,000         | 14,100      |
| 15         | 34,600         | 13,700      |
| 39         | 33,800         |             |
| 44         | 34,600         | 24,200      |
| 16         | 28,000         |             |
| 42         | 28,300         | 14,600      |
| 12         | 14,300         | 23,800      |
| 7          | 23,700         | 23,900      |
| 27         | 24,000         | 23,600      |
| 42         | 24,300         | 28,500      |

tures. Such a combination ( $\mu = 14,000 \pm$  from 15–20°C. and  $24,000 \pm$  from 20°C. upward) is, however, to be expected if there exists a genetic recombination of the various types of curves observed in the parent strain. It will be noted again that the values of  $\mu$  are *only* those values observed in the parent strains, though many other values "might" have been obtained (*cf.* Stier and Pincus, 1928, and Table I).

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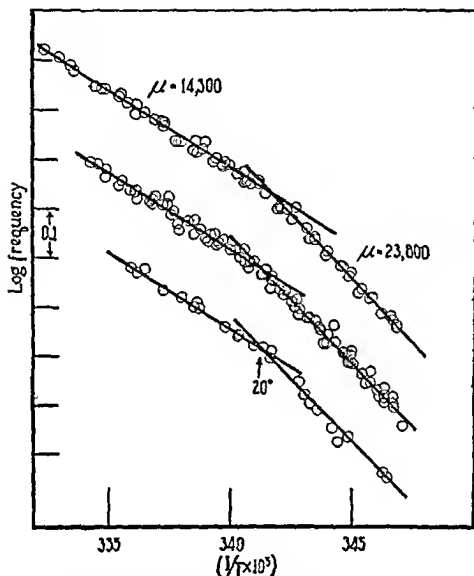


FIG. 6. Upper curve: Data from five individuals of the  $F_1$  generation which gave the same values of the temperature characteristic, namely,  $24,000 \pm$  from  $15-20^\circ\text{C}$ . and  $14,000 \pm$  from  $20^\circ\text{C}$ . upwards. Middle curve: Data from nine individuals of the backcross generation  $F_1 \times \text{albino}$ .

Lower curve: data from three individuals of the backcross generation  $F_1 \times \text{dilute brown}$ . The lines drawn through the data for the two backcross generations are exactly parallel to those drawn through the  $F_1$  data. (No attempt is made to distinguish individuals by separate symbols.)

taken on successive occasions. The existence of a critical temperature at  $20^\circ\text{C}$ . is demonstrable in one instance (middle curve) by an abrupt shift in the rate as well as by a change in the value of the tem-

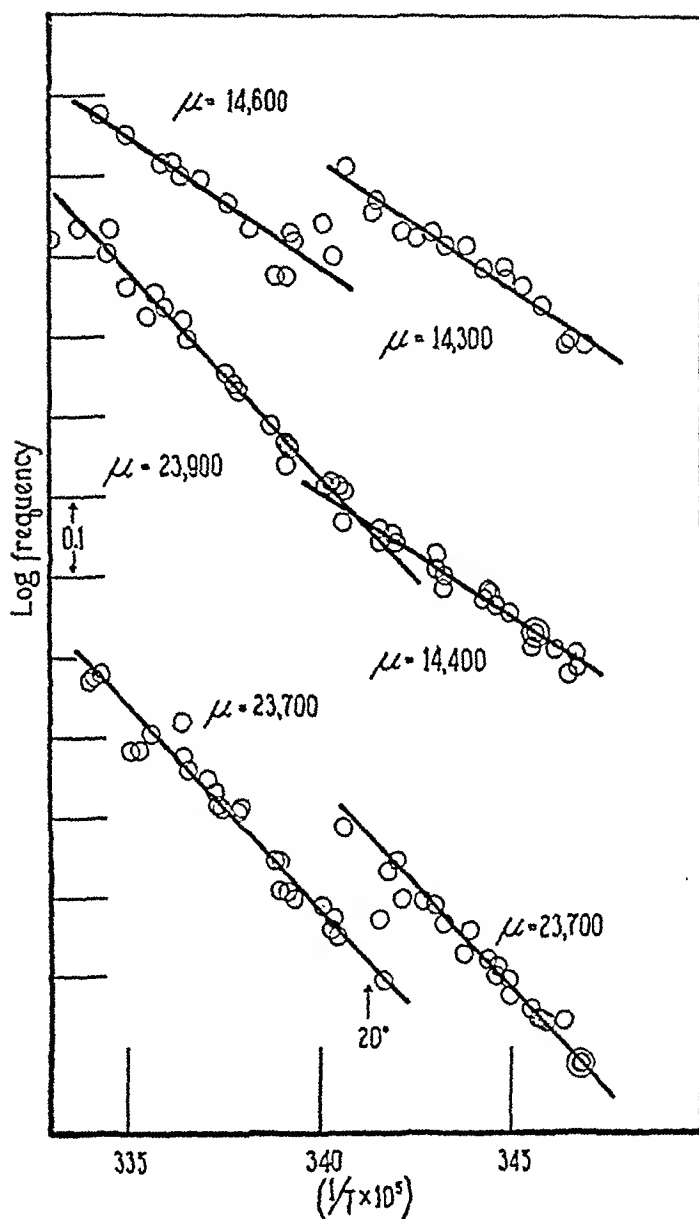


FIG. 7. Upper curve: Data from three individuals of the backcross  $F_1 \times \text{albino}$ , illustrating the nature of the "shift" at  $20^\circ\text{C}$ . (cf. lower curve).

Middle curve: Data from four individuals of the backcross  $F_1 \times \text{dilute brown}$  and from one individual of the backcross  $F_1 \times \text{albino}$  illustrating a recombination of two parental types of curve.

Lower curve: Data from three individuals of the backcross  $F_1 \times \text{dilute brown}$  and from two individuals of the backcross  $F_1 \times \text{albino}$ , showing the "shift" at  $20^\circ\text{C}$ . in association with values of  $24,000 \pm$  of the temperature characteristic.

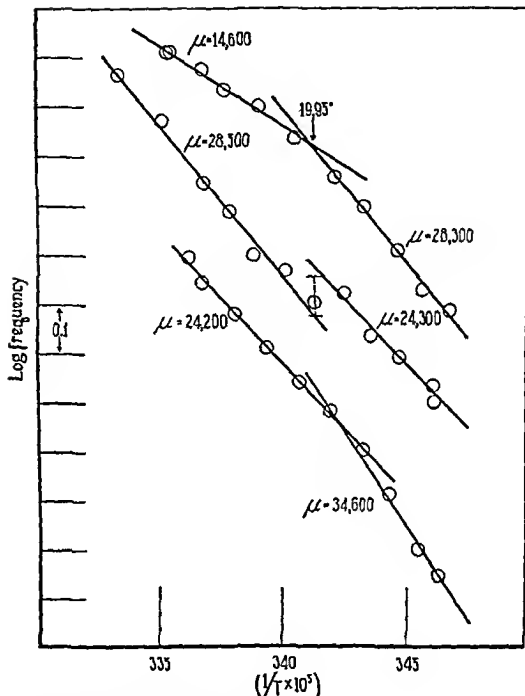


FIG. 8. Data for three backcross individuals.

Upper curve: Mouse No. 42, 8 days old.

Middle curve: Mouse No. 42, 6 days old. Note critical temperature ( $19.95^{\circ}\text{C}.$ ) at same temperature in each case, but expressed by a "break" in the upper curve and by a "shift" in the middle curve. Horizontal bars above and below the point at  $19.88^{\circ}\text{C}.$  (middle curve) represent the two modes of a bimodal frequency distribution for the data taken at that temperature.

Lower curve: Mouse No. 44 F,  $\times$  albino generation. See text.

perature characteristic. In the upper curve, on the other hand, there is no shift but simply a change in value of the temperature characteristic. Here also one sees in an individual what is observed in the data as a whole,—namely, the occurrence of typical though not the same values of the temperature characteristic over the temperature ranges used. The lower curve illustrates how data for a single

TABLE VI

| Animal no.               | Temperature         | 100/T  | Average time<br>for five beats<br>(first thirty<br>readings) | Probable error<br>$\pm$ | 100 P E<br>mean |
|--------------------------|---------------------|--------|--|-------------------------|-----------------|
|                          | $^{\circ}\text{C.}$ |        |  |                         |                 |
| <i>Dilute brown</i><br>1 | 15.25               | .34680 | 12.596   | 0.204                   | 1.62            |
|                          | 16.43               | .34539 | 10.463   | 0.191                   | 1.81            |
|                          | *17.57              | .34404 | 8.573  | 0.113                   | 1.31            |
|                          | 18.16               | .34334 | 9.130  | 0.147                   | 1.61            |
|                          | 20.13               | .34103 | 6.586  | 0.104                   | 1.58            |
|                          | 21.85               | .33904 | 5.126  | 0.085                   | 1.66            |
|                          | 23.54               | .33711 | 3.920  | 0.068                   | 1.73            |
|                          | 24.92               | .33555 | 3.400  | 0.064                   | 1.88            |
|                          | 25.95               | .33440 | 3.166  | 0.054                   | 1.70            |
|                          | 26.92               | .33331 | 3.906  | 0.105                   | 2.69            |
| <i>Backcross</i><br>8    | 15.66               | .34631 | 7.506  | 0.104                   | 1.38            |
|                          | 16.90               | .34483 | 5.636  | 0.083                   | 1.46            |
|                          | 17.47               | .34415 | 4.973  | 0.073                   | 1.46            |
|                          | 18.60               | .34282 | 4.126  | 0.054                   | 1.30            |
|                          | *18.89              | .34248 | 3.733  | 0.040                   | 1.06            |
|                          | 19.86               | .34134 | 3.493  | 0.041                   | 1.17            |
|                          | 21.40               | .33956 | 3.050  | 0.033                   | 1.08            |
|                          | 22.86               | .33788 | 2.566  | 0.029                   | 1.11            |
|                          | 24.61               | .33590 | 2.300  | 0.025                   | 1.09            |
|                          | 25.92               | .33443 | 1.993  | 0.023                   | 1.16            |

\* First temperature.

individual are very well fitted by curves from which the typical values of the constant  $\mu$  may be calculated.

That the lines fitted to the observed frequencies are justified is borne out not merely by the way the points "hew to the line," but also by a consideration of the variability of the data. It has been pointed out (*cf.* Crozier, 1929; Navez, 1930; Stier, Pincus, and Crozier, 1931) that adequate data taken over a range of temperatures and de-

scribed by the Arrhenius equation should show a constant coefficient of variation, and that the probable errors of the mean observations should change in the same way with temperature as the means. This is in fact the case with these data. A statistical study of this variation is considered elsewhere (*cf.* Stier, Pincus, and Crozier, 1931), but a concrete illustration of the nature of this independent test of the goodness of fit of the lines drawn to the present data is given in Fig. 9. The data for Fig. 9 are given in Table VI. The last column of Table VI gives a "coefficient of variation". In the case of *dilute brown* animal No. 1 note the obvious increase in variation beyond the critical point at 25°. The points 15°66 to 18°90, clearly below the critical point in the case of backcross animal No. 8, give an average value of 1.400 for the ratio  $\frac{100 P E}{\text{mean}}$ ; those above the critical point give an average value of 1.122. This difference is significant and indicates that they belong in separate categories. One point (at 18°89) below the critical temperature represents the first temperature used in the experiment and falls on the line drawn for points above the critical temperature. The value of the ratio  $\frac{100 P E}{\text{mean}}$  for this point is 1.06 and falls in with the average values for points on that line (for another case see Crozier, 1929, p. 97).

It is also pertinent to remark that where a critical temperature occurs, one may find in a set of data taken just above or below the critical temperature, a bimodal distribution of the frequencies representing frequencies typical of now one, now the other curve. The mean value from such data would lie between the two curves. The point at 19°98 in the middle curve of Fig. 8 illustrates such a situation, the two modes observed in the data being represented by the horizontal lines. The mode for the lower frequencies lies exactly on the lower line ( $\mu = 28,500$ ), whereas the mode for the higher frequencies lies below the upper line ( $\mu = 24,300$ ). This meets expectations exactly, inasmuch as certain respiratory heats proper to the lower frequencies might well be included in readings containing chiefly heats of higher frequency, and inclusion of heats of high frequency in readings of lower frequencies is not to be expected so often since the mean is so close to that to be expected from the lower line.

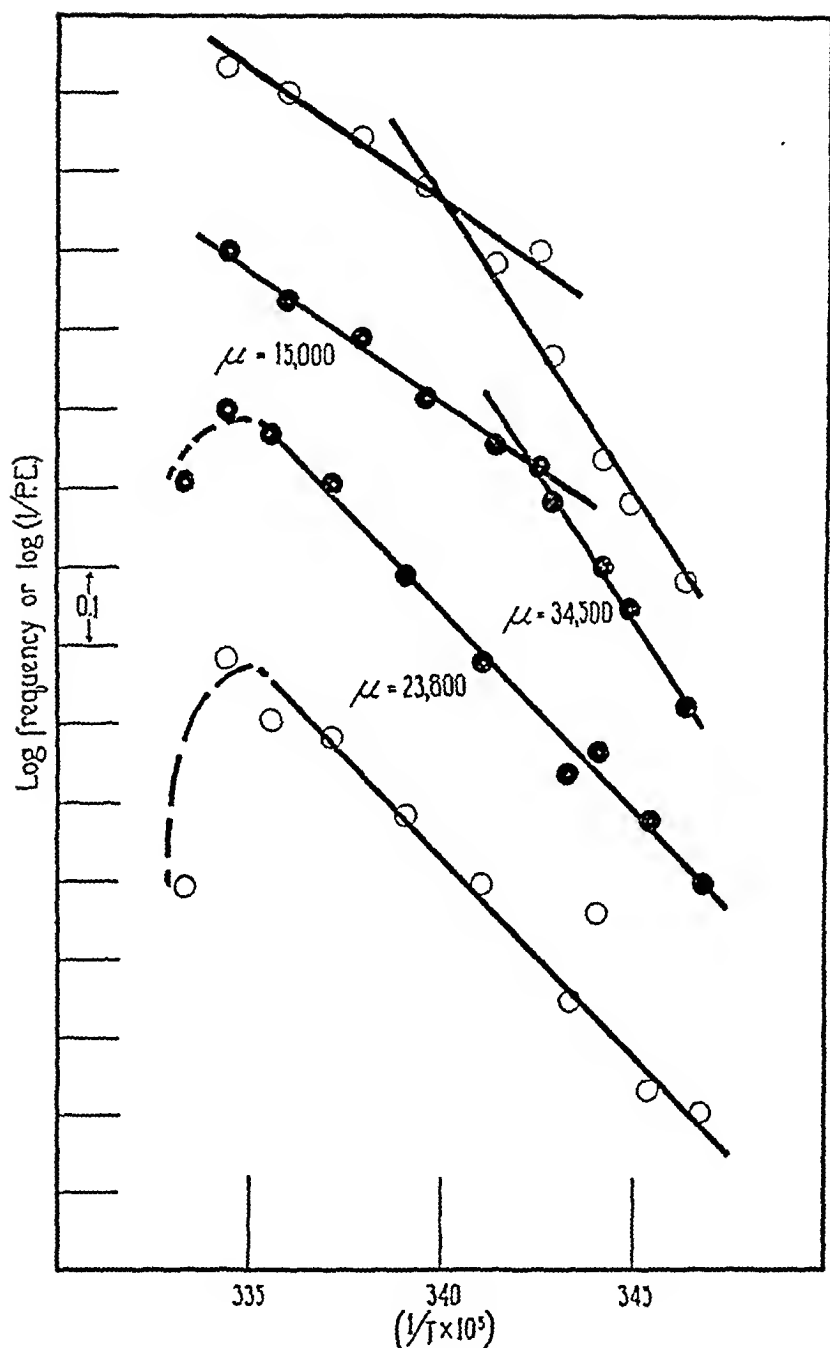


FIG. 9. Closed circles represent data on breathing movements; each point is the mean of the first thirty readings. Open circles represent the cologarithms of the probable errors at each temperature. Upper two curves from *backcross* animal No. 8. Lower two curves from *dilute brown* animal No. 1. The curves drawn through the open circles are exactly parallel to those drawn through the closed circles. The one point markedly off (at  $17^{\circ}57$ ) in the lowest curve is from readings taken at the first temperature studied; such points are often "aberrant." See text and Table VI.

## V

There are indications of genetic segregation of certain of the relations of breathing-frequency to temperature, but data are as yet insufficient for any thorough genetic analysis. One observes, for example, a critical temperature at about 20°C. in all the *albinos* tested, but in none of the *dilute browns* tested. Eleven out of twelve  $F_1$  animals show this critical temperature, all backcross animals out of the cross  $F_1 \times \text{albino}$  show it, and fifteen out of twenty-two animals of the cross  $F_1 \times \text{dilute brown}$  show it. The implication is that this critical temperature is dominant and that segregation occurs in the expected backcross ( $F_1 \times \text{dilute brown}$ ).

Again, one observes a sort of segregation of the constant  $\mu$ . Over the range 15–20°C. most of the  $F_1$  animals (ten out of twelve) show the value  $\mu = 24,000 \pm$  or  $28,000 \pm$ , which is the *dilute brown* type. Similarly, in the range 20°C. upward, eight out of ten animals show the value  $\mu = 14,000 \pm$ , which is the *albino* type. If the value  $\mu = 24,000 \pm$  or  $28,000 \pm$  were "dominant" over the range 15–20°C., and if this "dominance" were due to a single Mendelian factor, we would expect in the backcross  $F_1 \times \text{albino}$  approximately equal numbers of the *dilute brown* type ( $\mu = 24,000 \pm$  or  $28,000 \pm$ ) and the *albino* type ( $\mu = 34,000 \pm$  or  $14,000 \pm$ ). Actually we observe fourteen individuals showing the *dilute brown* type to eleven showing the *albino* type. If over the range 20°C. upward the *albino* type ( $\mu = 14,000 \pm$ ) were "dominant" we would expect all individuals to show this value in the cross  $F_1 \times \text{albino}$ . Actually eighteen out of twenty-three individuals show this value, five giving the value  $\mu = 24,000 \pm$ . The latter five are from any strict Mendelian standpoint "exceptional" individuals. In a like manner one finds indications of segregation in the backcross  $F_1 \times \text{dilute brown}$ , but again six "exceptional" individuals are observed. There is, therefore, neither in the  $F_1$  or backcross generations any simple Mendelian behavior. As has been pointed out, one need not necessarily expect results conforming to the elementary Mendelian scheme, but the suggestion of an approach to such behavior opens interesting possibilities. Speculation in this direction is deemed unwise until a more exhaustive analysis is undertaken.

The writer wishes also to defer discussion of the literature in the



field in which this investigation represents a special phase. It may be pointed out, however, that the type of approach further exemplified in these experiments offers a unique method for unearthing and investigating otherwise undetectable physiological differences, and has special advantage in that it permits the study of the genetics of functional relationships in single individuals.

The occurrence in the  $F_1$  and backcross generations of only those values of the temperature characteristic found to be exhibited by the parent strains has a peculiar significance for the theory of temperature characteristics. Crozier (1926, *etc.*) has analyzed a great variety of biological reactions involving temperature relations, and has found that the temperature characteristics derived exhibit a number of "modal" values. The values derived from the data of the present experiments fall into certain of these modal groups. Whether such modal values have any specific physico-chemical significance is as yet undetermined, but it cannot be questioned that the results of these experiments indicate that the particular values here observed have reality in a biological sense. For one can conceive of no more rigid test of the biological integrity of a process than that its descriptive constants are recoverable in successive generations of crossbreeding (*cf.* Pincus and Crozier, 1929; Crozier, Stier and Pincus, 1929).

#### SUMMARY

Young mice of a selected line of the *dilute brown* strain of mice exhibit over the range 15–25°C. (body temperature) a relation of frequency of breathing movements to temperature such that when fitted by the Arrhenius equation the data give a value for the constant  $\mu$  of  $24,000 \pm$  calories or, less frequently,  $28,000 \pm$ . Young mice of an inbred *albino* strain show over the range 15–20°C. a value of  $\mu = 34,000 \pm$ , or, less frequently,  $14,000 \pm$ , with a critical temperature at about 20°C. and a value of  $\mu = 14,000 \pm$  above 20°C. The  $F_1$  hybrids of these two strains, and the backcross generations to either parent strain, exhibit only those four values of the temperature characteristic observed in the parent strains and none other. One may therefore speak of the inheritance of the value of the constant  $\mu$ , but the inheritance shows in this instance no Mendelian behavior. Furthermore there appears to be inherited the occurrence (or absence) of a critical

temperature at 20°C. These experiments indicate the "biological reality" of the temperature characteristics.

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# THE PHYSIOLOGICAL PRINCIPLE OF MINIMUM WORK

## A REPLY

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Columbia University and the Presbyterian Hospital, New York)

(Accepted for publication, December 11, 1930)

P. S. Bauer<sup>1</sup> has recently questioned the validity of the Physiological Principle of Minimum Work as formulated and applied by me<sup>2</sup> to certain problems concerning the efficiency of operation of the oxygen transport system in man.

In contrast to his opinions or the implications which he makes in respect to my work, I reply that this principle should not, and does not rest upon any principle of physics which applies, like the Principle of Least Action (no mention of which was made in my papers), exclusively to ideal conservative systems. Poiseuille's law of flow was used as the simplest approximation to conditions obtaining in capillaries, and the gravitational factor (especially for a man in the basal state and lying down) was neglected as one of secondary magnitude compared to those dealt with in the general problem. Some elementary procedures in calculus, mechanics, and graphical analysis were used in solving the purely mathematical portions of certain problems, but these were simply the ordinary working tools of applied mathematics. Although supported by many analogies and general experience, the validity or "justification" of the Physiological Principle of Minimum Work rests primarily upon the approximate consistency established between theory and certain facts of observation.

The principle merely states that in many cases and especially in the circulation of the blood the cost of actual operation of living systems tends to be a minimum and that cost is to be measured in units, like the calorie and the erg, which have dimensions equivalent to those of *work*.

<sup>1</sup> Bauer, P. S., *J. Gen. Physiol.*, 1929-30, 13, 617.

<sup>2</sup> Murray, C. D., *Proc. Nat. Acad. Sc.*, 1926, 12, 207, 299; *J. Gen. Physiol.*, 1926-27, 9, 835.



# ON CHROMOSOME BALANCE AS A FACTOR IN DURATION OF LIFE

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(Received for publication, December 16, 1930)

The earliest census returns showed that males in the human species live a shorter duration of time than the females. These sex differences in life span are found in a wide variety of races and world wide environmental conditions. In the United States Life Tables for 1910 Glover presents the average expectations of life for thirteen countries in eleven age groups up to the age of eighty years. To facilitate a clear-cut quantitative grasp of the problem as distinguished from the qualitative one we may quote these figures for the expectation of life in years at the first year of life. The male duration of life is given first followed by that of the female. In Australia the male expectation of life is 55.2 years and the female 58.8 years; Denmark 54.9-57.9; England 48.5-52.4; France 45.7-49.1; Germany 44.8-48.3; Holland 51.0-53.4; India 22.6-23.3; Italy 44.2-44.8; Japan 44.0-44.9; Norway 54.8-57.7; Sweden 54.5-57.0; Switzerland 49.3-52.3; and in the United States 49.3-52.5. The males live throughout a shorter time than the females. This difference persists under conditions as diverse as those from the north temperate zone to the equator, under as varied public health administrations, food conditions, and population densities as lands under cultivation and civilization for unknown time to lands first broken by the plow within the memory of living men, and finally with peoples of long widely separated racial origin. This difference in the mean life span of the two sexes likewise holds for much of the animal kingdom.

It is natural to ask what may be the cause of these sex differences in duration of life. *Drosophila* is in many ways exceptionally well fitted to the study of such problems, as well fitted to population studies perhaps as the albino rat is for nutrition or the rabbit for

immunology. It has been intimately studied for its duration of life under a number of strictly controlled experimental conditions. Pearl and his coworkers have presented life tables showing the duration of life of the separate sexes for several races, food conditions, densities of population, temperatures, etc. Under all of these conditions and races the relative duration of life of the males *vs.* that of the females was surprisingly alike in one respect. The males lived a shorter time than the females. As a typical result of these studies it was shown that the duration of life of the normal wild type fly was 45.8 days for the males and 48.0 days for the females. The difference in the expectation of life of the males and females then holds for these insects as it does for man. This result is true even for such racially diverse strains as those of vestigial where the life span is markedly affected by inheritance. In an experiment on this race it was found that the male duration of life was 14.1 days and the female 19.8 days. Despite the pronounced dissimilarity in the life of these two groups standing in a ratio of about 3 to 1 and attributable to the inheritance of the two races, the sexes still show this marked difference, the female living the longer life. In another experiment where the food condition was the major variable the sex difference in life duration held in much the same degree as under the conditions of full feeding, the difference being about 10 per cent. Under temperature variations as extreme as those from 18–28°C. the sex differences in life still held although life at the cold temperature moved only about half as fast as it did in the hot climate as measured by life rates of 43 days and 71 days for the cold temperature and 23 days and 29 days for the hot temperature, male duration of life being given first.

The differences in duration of life of the sexes is as common for the fly *Drosophila* as it is for man. What it may be asked is the physiological basis for these differences? Many suggestions have been offered to explain the difference. Some have attributed it to a difference in the environment in which the sexes live, males being pugnacious by nature are exposed to sudden death more frequently than the female, or again since in the nature of things the male is to get the food in the struggle for survival he is subject to economic vicissitudes and their attendant physical and mental stresses to a greater extent than the protected females. From the point of view of another group, man in

his search for food for himself or his industries is exposed to more diseases than the female, thus favoring the males' earlier death. Unfortunately, plausible as these and many other suggestions are for man, they do not hold for *Drosophila* kept under carefully controlled conditions where both sexes have not only an equal chance but an optimal chance to show their full potentialities for life. Yet the difference in life span exists in as marked a degree. There would appear to be something much more important than environment or at least deeper in life's structure to account for the observed differences. A moment's consideration of the physical basis of sex may indicate what this factor differentiating the life of the two sexes may be.

Current research in modern biology has come to the view that sex is a resultant of interacting forces generated by the balance of genes within the so-called sex chromosomes and the other chromosomes of the group, the autosomes. The male balance in *Drosophila* and probably in man is determined by one sex chromosome of the X-type and the two sets of autosomes. The female balance appears to be the resultant of two sex chromosomes of the X-type and the two sets of autosomes characteristic of the species. This differentiation goes back to the egg. It initiates the chain of events which normally leads to the adult sex differences. In this paper attention is called to these chromosome differences as a possible explanation of the differences in the duration of life of the sexes. This hypothesis may be approached in *Drosophila melanogaster* because under certain conditions other forms than those producing the normal male and female complex of chromosomes are produced. These forms are (1) triploids having three sex chromosomes and three of each chromosome of the autosome group and (2) sex-intergrades having two sex chromosomes of the X-type and generally a Y-chromosome, and three of each chromosome of the autosome group.

The triploid is thus just like the normal female (two sex chromosomes of the X-type and two of each chromosome of the autosome group) save that it has added to its chromosome complex a full haploid group of chromosomes, (one X-chromosome and one each of the autosome group). The balance between the sex chromosomes and autosomes is therefore the same as in the normal female. The duration of life of these triploids gives a test of the influence of an extra set



of chromosomes when the whole is in full balance. The second group of abnormal forms, the sex-intergrades, furnish a group with extra chromosomes but with the X-chromosomes unbalanced as compared with the autosomes. The triploids furnish a test of the effect of the extra chromosomes in balance, and the sex-intergrades furnish a test of the influence of the unbalance between the X-chromosomes and the autosomes. Should it be that the normal male's duration of life is shortened by the unbalance of his single X-chromosome with the two sets of autosomes, then by parallel reasoning it would be expected that the sex-intergrades would likewise have a shorter duration of life than the triploids. If the facts actually observed fit this reasoning they become proof for the theory that the difference in the duration of life of the sexes is due to differences in chromosomes number and therefore gene balance.

### *Material and Methods*

The material from which these stocks of flies were derived came from a race bred some 300 generations in my laboratory, known as the cx. stock. This stock has the property of the females producing diploid eggs which on fertilization give triploid flies. Triploid flies from these stocks were selected in the course of observing these cultures. They were then bred to the inbred stock of cx. flies for a number of generations. After the expiration of not less than five generations of such matings individual bottle matings were made and from these bottles the four classes of flies were selected having the following cellular characteristics:

|                        | Sex chromosomes |     | Auto-<br>somes | Condition  |
|------------------------|-----------------|-----|----------------|------------|
| Type males. ....       | 1 X             | 1 Y | 6              | unbalanced |
| Type females. ....     | 2 X             |     | 6              | balanced   |
| Triploid females. .... | 3 X             |     | 9              | balanced   |
| Sex intergrades. ....  | 2 X             | 1 Y | 9              | unbalanced |

The technique was as follows: The parental triploids for these experiments were mated singly and placed in the ordinary half-pint milk bottles on the standard corn meal—Karo—molasses—salts and yeast media. The cultures were incubated at 22° until they were about to hatch. The bottles were examined and all hatched flies removed within 24 hours after hatching. The freshly hatched flies were examined for triploids. The triploids were the least frequent class. The sex-intergrades, normal males and females followed in frequency in the order named. The two classes of especial interest to the experiment were the triploids and sex-intergrades. If either triploids or sex-intergrades were present a random sample

of the other classes was made from the flies from this same bottle in number approximately equal to the triploid or the sex-intergrade classes. These samples of the different classes were placed in separate vials one inch by four inches fitted with a stopper perforated with a large hole covered by copper screen and carrying a glass slide on which was kept a thin layer of the food medium plus yeast. These glass slides were changed every other day throughout the life of the flies in the bottle. These vials were always kept next to each other in the racks at a temperature of 22°. Counts of the deaths were made daily.

As the chance of finding more than a very limited number of triploids per bottle on any one day is slight it follows that the density of flies per duration of life bottle was low. Furthermore, since two classes were of interest the classes could not be kept identical in number. Pearl and Parker have shown that a density less than thirty-five flies per bottle is detrimental to a long duration of life. Our initial densities varied from one to ten per bottle. The average density was 4.9 flies for the normal females, 4.2 flies for the normal males, 2.4 flies for the triploid females, 5.1 flies for the sex-intergrades. The actual figures for the duration of life within one bottle would therefore be expected to be less than in bottles where the density is about the optimum, thirty-five flies. However, this effect is equally common to all classes of our flies. The result for our normal males and females when compared with those of Pearl and Parker show that in our experiment this factor density had the same order of effect as it did in their experiments.

### *Survivorship Curves*

The data are plotted as the number of individuals surviving at the beginning of each indicated age period, on the basis of 1000 flies starting together at immurgence. The whole number is given in each case. The raw data are given, no effort being made to smooth the results.

These distributions and the graphical presentations of them in Fig. 1 show at once that the groups are markedly differentiated. The features distinguishing these groups have as their ultimate cause the chromosome differences found within their cells. These chromosomes are balanced or unbalanced. This chromosome balance in turn influences the balance of the genes since the chromosomes are the carriers of the genes.

The normal females show the longest duration of life as evidenced by the fact that their life line is the highest of the diagram. The triploid females show practically the same duration of life as that for the normal diploid females. This fact is significant since these two types of flies have their chromosomes in balance three sex chromosomes to three sets of autosomes and two sex chromosomes to two sets of



If all the curves be examined it will be noted that the sex-intergrade curve differs from the rest in that it presents the form of a straightline diagonal of the chart. The instantaneous death-rate is thus a constant at all ages from the time of immergence as the imago to the death of the last fly.

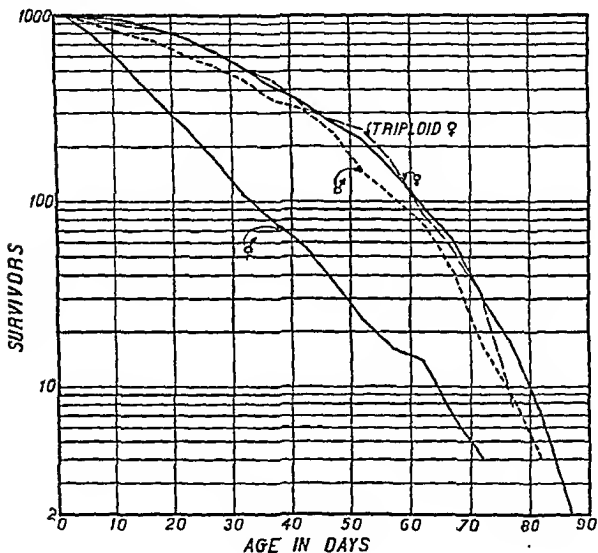


FIG. 1. Survivorship distribution of *Drosophila* for individuals with different chromosome groups.

The other three curves are about midway between this type and the other theoretical type of a right angle curve where all the individuals emerging from the pupa live to the same age when they all die. These *Drosophila* curves are thus closely similar to the many curves published on other *Drosophila* material and on man.

Table II shows the physical constants of variation for the duration of life within the four groups. The most noticeable fact about this tabulation is that the normal females and the triploid females lived the same average length of time. Both of these flies are females in every sense (Gowen, 1930). The males lived  $4.2 \pm 1.0$  days less than the normal female and triploid classes. Qualitatively and also quantitatively this difference is comparable to that found in many studies, the males living a tenth or so less time than the females. The sex-intergrade class live the least time of all, in fact only half the life of the type males, females, and triploids. The unbalance of the chromosomes makes the type males differ from females in the features by which they are normally recognized. The expression of these differences is found in the length of the life span just as in the more

TABLE II

*Constants of Variation for Life Span in Drosophila, Balanced and Unbalanced Chromosome Cohorts*

|                      | Mean          | Standard deviation | Coefficient of variation |
|----------------------|---------------|--------------------|--------------------------|
| Type females.....    | $33.1 \pm .6$ | $17.9 \pm .4$      | $54.2 \pm 1.6$           |
| Type males.....      | $28.9 \pm .8$ | $19.0 \pm .6$      | $65.7 \pm 2.8$           |
| Triploids.....       | $33.1 \pm .8$ | $18.4 \pm .5$      | $55.6 \pm 2.1$           |
| Sex-intergrades..... | $15.0 \pm .3$ | $11.6 \pm .2$      | $77.3 \pm 2.3$           |

familiar characteristic differences in the primary and secondary sexual organs.

The fact that the type females and triploids are female throughout and live the longest time might be held to indicate that it was the presence of the female organs as such that resulted in the longer life of the females as contrasted with that of the males. This question can in a measure be tested by the sex-intergrade class. The individuals within this class are all of one chromosome constitution, two sex chromosomes and three sets of autosomes. A wide variability in the degree of development of the sex organs exists within this group (Gowen, 1930). Some individuals are externally almost perfect males or perfect females. Other individuals are mixtures of the organs found in either sex. The influence of the presence of the particular

type of sex organ complex on the life span may be tested by dividing the sex intergrade class into two groups, those which are most nearly like the female and those which are most nearly like the male. The individuals will have the same chromosome complex in the two groups but will differ in the development of the organs. The data here presented have been divided into these two groups designated as female appearing sex-intergrades and male appearing sex-intergrades. A wide variability still exists in each group. While the sex organs systems are not as well developed as in the type individuals, the groups

TABLE III  
*Survivorship Table for Drosophila Sex-Intergrades*

| Age in days | Male sex-intergrade | Female sex-intergrade |
|-------------|---------------------|-----------------------|
| 0-4         | 1000                | 1000                  |
| 5-9         | 828                 | 709                   |
| 10-14       | 652                 | 457                   |
| 15-19       | 483                 | 275                   |
| 20-24       | 362                 | 178                   |
| 25-29       | 258                 | 108                   |
| 30-34       | 181                 | 59                    |
| 35-39       | 142                 | 35                    |
| 40-44       | 112                 | 21                    |
| 45-49       | 78                  | 9                     |
| 50-54       | 56                  |                       |
| 55-59       | 39                  |                       |
| 60-64       | 35                  |                       |
| 65-69       | 17                  |                       |
| 70-74       | 9                   |                       |

as such are distinctly different from each other. The duration of life data and curves of the same are shown in Table III.

The pictorial presentation of these results is given in the second chart.

The survival lines of the male sex-intergrades are markedly higher than the survival lines of the female sex-intergrades. The result is therefore just opposite to that found for the type males and females. The conclusion appears to be sound that the differences observed between the sexes are not due to the sex organs in themselves. The average duration of life of the two groups is  $12.7 \pm .3$  days for the fe-

male sex-intergrades and  $18.4 \pm 7$  days for the male sex-intergrades. A wide variability in organ development still remains within these two groups, due apparently to the fact that the organism has not as yet adapted itself to a chromosome unbalance of two sex chromosomes and three sets of autosomes. To still further reduce this variability a portion of the sex-intergrades were divided into three classes, female

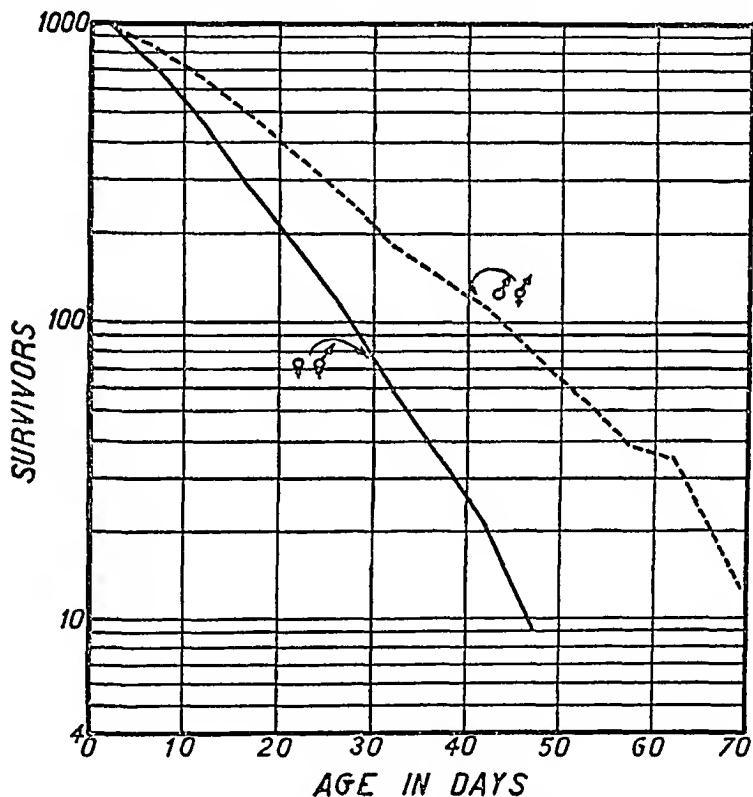


FIG. 2. Survivorship distribution of *Drosophila* sex-intergrades, the chromosome complex is the same but the development of the sex organs is unlike.

sex-intergrades containing most of the individuals like those of the female sex-intergrades given above, mid sex-intergrades having mixtures of the primary and secondary organs of both sexes and male sex-intergrades having organs like the males. The average duration of life in the three groups for a much less number of individuals was  $14.1 \pm 2.3$  days for the female sex-intergrades,  $17.7 \pm 1.3$  days for the mid sex-intergrades, and  $19.1 \pm 1.0$  days for the male sex-intergrades.

The facts support the data of the other experiment, that it is not the male or female sex system as such but rather the much more extensive alterations brought about by the relative balance of the sex chromosomes and autosomes which accounts for the difference in the duration of life of the sexes.

The forms brought about by the two sex chromosomes and three autosomes combinations are not suited to life in nature nor do they reproduce. The changes brought about by the unbalance of the chromosomes are much more severe on the animal than are the changes brought about by a still further unbalance of the chromosomes as seen in the type males. This fact has a distinct evolutionary interest since it suggests a cause for the trend of evolution away from the hermaphrodite toward the markedly bisexual species in the higher animals.

It may be noted in Fig. 2 that both types of sex-intergrade die at a constant percentage rate, a type of curve which has only been approximated in other forms. The death rate for the male sex-intergrades is approximately 6 per cent a day, the survival curve being

$$\text{Surviving male sex-intergrades} = A e^{-.064t}$$

when  $A$  = surviving individuals at any age in days and  $t$  equal age in days.

The female sex-intergrade curve shows a constant rate of death. This rate is nearly twice as great as that of the male sex-intergrade, being 10 per cent per day.

$$\text{Surviving female sex-intergrades} = A e^{-.103t}$$

The results herein are supported on rather meager data by those on two other groups of chromosome unbalanced flies, the super females with three sex chromosomes and two sets of autosomes and another unbalanced group having only one fourth chromosome. These results will be presented at a later date.

From another viewpoint, the facts brought out in this paper are of moment. The cells making up the bodies of the triploid and sex-intergrade are a third larger than the cells comprising the type males and females. They have more cytoplasm and chromatin. They must function in a bigger way than the ordinary cells since there is more material to be maintained and greater distances to be traversed. The



fact that the triploid females are able to live as long as the diploid females shows that the two types of cells are capable of functioning equally well.

### *Survival Curves in Other Species*

Survival curves have been determined for bacteria under a variety of conditions. The universe of experiment has been chiefly an unfavorable environment due to disinfectants or X-ray. Like curves have been determined for protozoa, worm, and *Drosophila* eggs, etc. From the form of the curve taken by the raw data of such experiments a theory for the cause of death of the particular form experimented with has generally been evolved. This approach to the problem has, on the whole, been interesting but sterile since it has led to dynamically opposite views, those which account the variability to the biology of the organism, and those which attribute it to chemical degeneration processes. It takes information independent of that derived from curve fitting to throw light on the physical meaning to be attached to the constants of the curves.

Pearl and his coworkers have performed such an analysis by showing that it is possible to take a species with a determined life curve and by changing the genetic constitution of the species to alter the life curve and to change its form. Biological variability of the species is consequently a demonstrated factor in the forms which life curves take in this species. On a less exact scale perhaps this fact has been demonstrated several hundred times in *Drosophila* for specific genes ranging all the way from genes which cut the life curve off in the egg to those which kill in the larva, pupa, or imago. That such genes are universally common is shown by their demonstration in mice, cattle, dogs, man, corn, wheat, barley, in fact in almost all forms of life in both the animal and vegetable kingdoms. The data of this paper further extend this principle of biological variation to another phase of the subject, namely the balance brought about in the organs by the chromosomes. This balance alters the effects of the different genes and markedly influences both the shape of the life curve and the duration of life for the different groups in which this species may be divided.

The fact that survival curves may follow those for a unimolecular, bi-, or various other reaction does not in itself prove that life is de-

pendent on a single, double, etc., molecular complex as many have assumed. It may equally well mean that some one organ, and this organ may have any complexity, takes on major importance to life within the particular experiment and that when exposed to the chances of being hit by the lethal agent, this organ will succumb with one, two, — hits, as the case may be. The observed curves need not and probably do not have more than a distant relation to single molecules as such.

It is significant to note that the *Drosophila* life curve has been shown in the study here presented to be capable of division into five different curves. The first is the familiar fact that while of the same general form, the type female life curve is about a tenth higher than the male life curve. The curve for the triploids is like that for the type females. These curves are of the type which can be generated by organs varying in their biological possibilities for life due to their innate characteristics. That this interpretation is correct is at least indicated by the fact that the other two groups, the female sex-intergrades and the male sex-intergrades show the type of life curve which would be generated by a single organ becoming of prime importance to life. Furthermore the organ which has this major vital rôle must be different in the two groups since the rate of degeneration is significantly different in the two curves.

#### SUMMARY

This paper presents a study of the influence of chromosome balance on duration of life in *Drosophila*. The balanced type of cells are shown to favor a longer life than are the unbalanced type.

Under the identical conditions of the experiment, the type females live an average of  $33.1 \pm .6$  days; the type males  $28.9 \pm .8$  days; the triploid females  $33.1 \pm .8$  days and the sex-intergrade females  $15.0 \pm .3$  days. The unbalance of the chromosomes and therefore of the genes contained within them is evidently a fundamental factor in the probable life span of the individual. The magnitude of the effect is fully on a par with that found for other factors, *i.e.*, different Mendelian genes for constitutional vigor, etc.

It has been possible to show by a study of the various sex classifications within the sex-intergrade class that the presence or absence of ovarian or testicular tissue as such is not the primary cause of the

difference in the life duration in the type males and females but that the cause is to be found deeper, sex determination and duration of life accompanying each other and resulting from the common cause, chromosome constitution.

The survival curve of the sex-intergrade groups present a limiting curve of duration of life, a constant death rate for each day of age. The curves have different rates of degeneration. To account for this fact it is necessary to assume that for these particular organisms a different organ in the two groups has assumed major significance to life due to the gene complex which causes their differentiation. Recurrent chance environmental and hereditary agents acting on organs generate the type of probability curve observed.

Triploid flies are made up of cells which are one-third larger than the cells of the type flies. It is not without significance to note that such individuals show no greater or less duration of life than do the ordinary flies when both groups have their chromosomes in balance.

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# METABOLISM AS RELATED TO CHROMOSOME STRUCTURE AND THE DURATION OF LIFE

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In this paper it is proposed to measure the katabolism of four fundamentally distinct groups of animals all within the same species and having closely similar genetic constitutions. These groups differ in what are perhaps the most significant elements of life. The chromosome structure of the first group is that of the type female, diploid; the second group is that of the type male, diploid but having one X and Y instead of the two X-chromosomes of the type female; the third group of flies are triploid, three sex chromosomes and three sets of autosomes; the fourth group, sex-intergrades has two X-chromosomes and three sets of autosomes.

Katabolism is a direct function of the cells composing the bodies of all animals. The cells of these four groups differ in size; the type male cells are the smallest; the females are somewhat, possibly a tenth, larger; the sex-intergrades and triploid cells are a half larger. The larger cell size suggests that any function within the cell would be performed in a larger way. The carbon dioxide production should enable us to measure physiologically the extent of this activity and present us with data on that fundamental point the relation of cell size to metabolic activity.

The durations of life of these different groups have been measured. The forms with the balanced chromosome complexes within their cells live the longest time, the unbalanced groups the least. Rubner has linked the duration of life with the amount of energy which the animal is capable of metabolizing. He states that the total energy transformed for a given body weight and for the whole life of the animal is an approximate constant for a large variety of animals. From this fact he elaborated what is perhaps the most complete theory to account

for the regulation of the duration of life as the time necessary to metabolize a given quantity of energy. Concrete and suggestive as this theory is, it has been tested to only a limited extent. Slonaker in an apparently well controlled experiment consisting of a group of 3 exercised rats *vs.* 3 litter mates which were not exercised obtained data which was interpreted as showing that those which exercised used their potential energy faster and died earlier than those which did not. The odds for significance of this conclusion are .015. Loeb and Northrop have shown that the duration of life of aseptic *Drosophila* cultures was a function of temperature. Since activity and therefore the metabolized energy consumed is also a function of temperature, Pearl has concluded that this data of Loeb and Northrop and also Slonaker's results are to be interpreted as favorable to Rubner's view. Northrop in a later experiment showed that the total CO<sub>2</sub> production of *Drosophila* cultures for their life time was 445 mg. at 15°C.; 411 mg. at 22–26°C. in light; 272 mg. at 26°C. in the dark; and 246 mg. at 30°C. in the dark. From these results Northrop draws a conclusion adverse to Rubner's theory.

The metabolic rates of the four groups of flies here described furnish a unique opportunity to obtain critical data on these problems. Qualitatively the results have favored the hypothesis that the production of CO<sub>2</sub> per mg. of body weight per 24 hours is a function of the chromosome balance and therefore of duration of life; quantitatively the relations are not exact.

### *Material and Methods*

The flies for this experiment came from the same stock as that previously used in the duration of life experiments. This cx. stock has the property of producing a limited number of triploid females. These triploid flies when bred have progeny of four types.

|                       | Sex chromosomes |     | Autosomes | Condition  | Mean duration of life |
|-----------------------|-----------------|-----|-----------|------------|-----------------------|
| Type males.....       | 1 X             | 1 Y | 6         | unbalanced | 28.9±.8               |
| Type females.....     | 2 X             |     | 6         | balanced   | 33.1±.6               |
| Triploid females..... | 3 X             |     | 9         | balanced   | 33.1±.8               |
| Sex-intergrades.....  | 2 X             | 1 Y | 9         | unbalanced | 15.0±.3               |

To obtain a sufficient number of each of these types, the primary triploid females were mated singly to the inbred males of this same stock. Triploids were selected

from the progeny to be again bred to the inbred stock, the process being repeated for at least seven generations. Progeny not older than 24 hours were selected from the eighth or more generation culture to be used for the determination of the carbon dioxide production over a 24 hour period. The number of flies in the triploid and sex-intergrade classes are few. The individual tests of carbon dioxide production were consequently performed on a limited number of flies per test experiment. While this difficulty has the very real effect of causing the deviation between experiments to be of wide ranges, it has a compensating and equally real advantage, it enables the investigator to be positive as to the exact number of flies alive during the experiment as well as at the beginning of the experiment and at the end of it.

The selection of flies to test was performed in the following manner. If either triploids or sex-intergrades were found in the hottle a like random sample of the type females and males was also made. These random samples of each group were divided so that where possible there were ten flies for the production of carbon dioxide in each test experiment.

The carbon dioxide determinations were carried on in vials 1 inch by 4 inches. 5 cc. of barium hydroxide one hundredth molar was placed in the bottom of the vial. A screen of coarse cloth was placed above the barium hydroxide. The flies to be tested were kept above this screen. The vial was closed by a rubber stopper vaselined to prevent any loss or gain of  $\text{CO}_2$  within the vial. This vaseline on the stopper proved to be a most important matter despite its simplicity. The rubber stopper of the vial carried a glass tube, open on the side, in which was kept a small quantity of cotton moistened with freshly boiled distilled water. No food of any sort was present in the vial. The vials were kept at  $20^\circ\text{C}$ . for the 24 hours during which the experiment was in progress. The flies were kept in the dark. Blanks were in duplicate or triplicate for each experiment. The blanks were quite uniform both with their checks and for the amount of  $\text{CO}_2$  absorbed from the air originally contained in the vial. The mean amount of this contained  $\text{CO}_2$  was  $.207 \pm .009$  mg. for the thirteen experiments. It was the intention to have all four classes of flies for each experiment, the sex-intergrades divided into three groups according to whether they resembled males, females or were largely intermediate between the two.

### *The Carbon Dioxide Production*

The results for the carbon dioxide production were all computed on a per mg. per 24 hour basis. The data for the individual experiments are shown in Table I.

The  $\text{CO}_2$  rates presented in Table I show that on the average the type females produce less carbon dioxide than any of the other groups. The triploid females produce slightly more carbon dioxide than the type females. The type males produce 25 per cent more carbon





dioxide than either the type females or triploid females. The sex-intergrade classes produce much more carbon dioxide than the type females or triploid females but are about equal to the type males in their metabolic rates. Within the sex-intergrade group the production of carbon dioxide is approximately the same for the three types.

The significance of these differences may be tested in a variety of ways according to the aspect of the data considered. If the standard deviation for the production of  $\text{CO}_2$  were calculated as was done in Table I the significance of the data is great. While the odds are less it seems more logical to regard the individual experiments as paired units in themselves. In fact the experiment as designed was intended for this method of testing for significance, "Student's" method. Each different type of fly was run with the other types in the same experiment and under identical conditions so far as known, the only exceptions to this occurring when the flies of a given group were missing from the cultures on that day. The material may therefore be justly treated as paired sampled material. This method of treatment shows that the type female class is throughout distinctly lower than the male class and the three sex-intergrade classes in its carbon dioxide per 24 hours per mg. of body weight. The type female class is identical with the triploid class in the amount of carbon dioxide produced. The males are significantly larger in their carbon dioxide production rates than the type females and triploid females. They are not differentiated from the mid, female or male sex-intergrade classes. The triploid females are like the type females, and markedly different from the males and the three sex-intergrade groups. The three sex-intergrade classes are comparable to each other and like the type male class; they are significantly different from the type females and triploid females. The probabilities from which these statements are derived are given on following page.

In another paper it was possible to show that these six classes of flies were also differentiated in regard to their duration of life. The mean life spans for the different groups were as follows: type females  $33.1 \pm .6$  days; type males  $28.9 \pm .8$  days; triploids  $33.1 \pm .8$  days; mid sex-intergrades  $17.7 \pm 1.3$  days; female sex-intergrades  $12.7 \pm .3$  days in larger group and  $14.1 \pm 2.3$  days in smaller sample; and male sex-intergrades  $18.4 \pm .7$  days in larger group and  $19.1 \pm 1.0$  days in

smaller group.<sup>1</sup> Of these six classes of inbred flies four are fundamentally different from the standpoint of chromosome structure. These six classes when kept under nearly identical environmental condition have the metabolic rates and durations of life indicated above. The comparison of these two variables for these six groups furnishes a uniquely critical test of Rubner's theory for the duration of life. Rubner's theory postulates an energy limit to the material which any animal may metabolize per unit of body weight. On reaching this limit the animal dies. Within the species *Drosophila* type females live longer than the males; the difference in average life

| Classes compared                                    | Odds  |
|---|-------|
| Type ♀ with type ♂.....                             | < .01 |
| Type ♀ with triploid.....                           | .04   |
| Type ♀ with mid sex-intergrade.....                 | < .01 |
| Type ♀ with female sex-intergrade.....              | < .01 |
| Type ♀ with male sex-intergrade.....                | < .01 |
| Type ♂ with triploid.....                           | < .01 |
| Type ♂ with mid sex-intergrade.....                 | .84   |
| Type ♂ with female sex-intergrade.....              | .10   |
| Type ♂ with male sex-intergrade.....                | .65   |
| Triploid with mid sex-intergrade.....               | .02   |
| Triploid with female sex-intergrade.....            | < .01 |
| Triploid with male sex-intergrade.....              | .01   |
| Mid sex-intergrade with female sex-intergrade.....  | .32   |
| Mid sex-intergrade with male sex-intergrade.....    | .25   |
| Female sex-intergrade with male sex-intergrade..... | .54   |

span being about 12 per cent. On the basis of Rubner's theory the males should metabolize their energy faster than the females. This they actually do, the difference in the rates being .036 mg. per day per mg. of body weight or a percentage difference of 27 per cent. Qualitatively the facts are in agreement with the theory, quantitatively the percentage difference is twice for the carbon dioxide rates and only once for the duration of life.

The triploids live the same length of time as the type females. They should consequently metabolize the same amount of energy.

<sup>1</sup> Gowen, John W., On chromosome balance as a factor in duration of life, *J. Gen. Physiol.*, 1930-31, 14, 447.

The data and theory are in essential agreement on this point. If the theory holds, the triploids should also use less oxygen and produce less carbon dioxide than the males and the three sex-intergrade classes. Here again the hypothesis and the facts concur qualitatively. Quantitatively the triploids live 12 per cent longer than the normal males and metabolize 23 per cent less carbon dioxide; a ratio of 1 to 2 instead of 1 to 1. The agreement is worse for the sex-intergrade classes. The mid sex-intergrades live 15.4 days or 43 per cent less time than the triploids. They metabolize 26 per cent more energy per day. The female sex-intergrades live 20.4 less days or 62 per cent less time and give off 14 per cent more  $\text{CO}_2$ . The male sex-intergrades live 44 per cent less time than the triploids and metabolize only 23 per cent faster.

The three sex-intergrade groups do not differ significantly in their carbon dioxide productions. What difference there is, however, is in the wrong direction. The female sex-intergrades metabolize the least and live the least instead of the most days as the theory would call for. The differences found for the intersex group should perhaps not be emphasized however because of the difference in the type of their life curves as compared with those of *Drosophila* as they are ordinarily known. These sex-intergrade survival curves are simple exponential curves with a constant rate of death for the male sex-intergrades of 6 per cent and for the female sex-intergrades of 10 per cent. They start at this rate from the initial class of the 1000 survivors. Such curves can be generated on the theory that some agent of death strikes by chance some special organ of paramount importance to life. The fact that the chromosomal organization of the sex-intergrade has so seriously affected some essential part of the organism as to cause the life curves to take the form they do, may equally well have altered the metabolic rates from what might be expected on the hypothesis of Rubner. The same excuse cannot be offered for the type female, male or triploid curves. Qualitatively these curves agree with Rubner's theory, quantitatively the agreement is imperfect. This irregular agreement is quite probably significant and to be interpreted as indicating not more than a partial truth in Rubner's theory.

These data may be summarized in slightly different form but emphasizing the same conclusions. Rubner's theory says that the energy



one produces a mechanical variation (Fig. 1*b*), whose curve begins like that in Fig. 1*a*, but is reversible and returns to the level from which

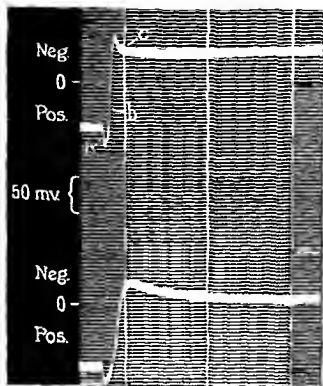


FIG. 2. Fast photographic record of an experiment arranged as in Fig. 3: the upper curve records the P.D. of *A* with reference to *C*, the lower curve that of *B* with reference to *C*. Cutting at *Q* produced the movements *a*, *b*, and *c* followed by others not shown on the record but indicated in Fig. 1*a*.

The intervals between vertical lines represent 0.1 second.

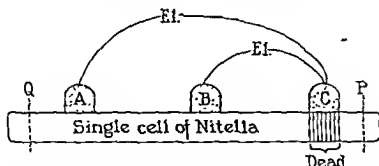


FIG. 3. Diagram to show the arrangement of an experiment. The spot *C* was killed by chloroform before the stimulus was applied: 0.001 *M* KCl was applied at all contacts.

it started without going above zero. The curve of the propagated negative variation (Fig. 1*c*) commences with an upward instead of a

downward movement, and this starts upward less abruptly than in the other cases (cf. Fig. 4).

Passage beyond a killed spot is illustrated by Fig. 5 which may be interpreted as follows: Cutting<sup>3</sup> at *X* (Fig. 6) resulted in a vigorous mechanical disturbance which traveled down the cell to *A*, and caused the irreversible change (death wave) shown by the upper curve (which does not return to the level from which it started). The disturbance passed the killed spot (*C*) but its intensity fell off as it progressed, and on reaching *B* it produced only the reversible response (mechanical variation) shown by the lower curve.

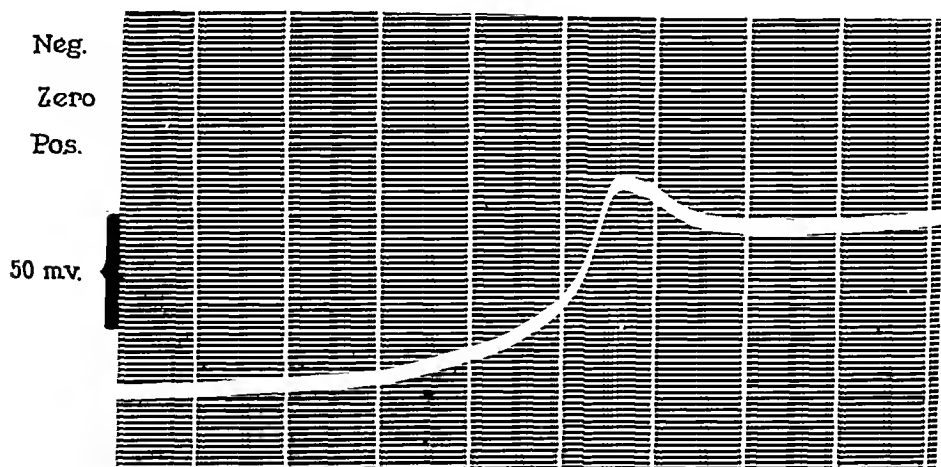


FIG. 4. Fast photographic record of an experiment arranged as in Fig. 3 with contacts *A* and *C* only. No mechanical stimulation but only a propagated negative variation (originating at *Q*), showing the gentle upward slope of the first movement *m*. Cf. Fig. 1 *c*.

The interval between the vertical lines is 0.2 second.

Such falling off is characteristic of mechanical disturbances: for example, even when the death wave does not pass a dead spot there is a loss of intensity as it travels.<sup>3</sup> This is shown in Fig. 7 (the loss is often very much greater). Both the amplitude and the speed of the

<sup>3</sup> Osterhout, W. J. V., and Harris, E. S., *J. Gen. Physiol.*, 1928-29, 12, 167, 355; Osterhout, W. J. V., and Hill, S. E., *J. Gen. Physiol.*, 1930-31, 14, 385.

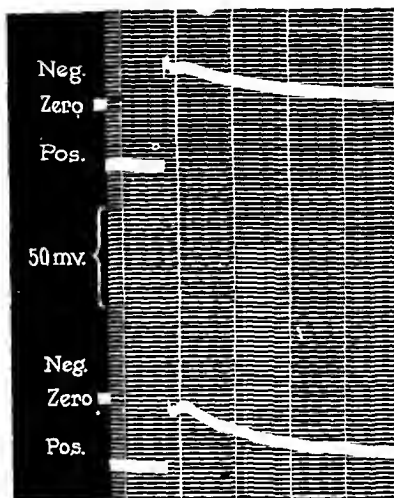


FIG. 5. Photographic record of an experiment arranged as in Fig. 6. The upper curve records the p.d. of *A* with reference to *C*, the lower curve that of *B* with reference to *C*. When the cell was cut at *X* (Fig. 6) a mechanical disturbance traveled to *A* producing an irreversible response (upper curve), continued past the dead spot (*C*), and produced a reversible response at *B* (lower curve).

The interval between the vertical lines is 5 seconds.

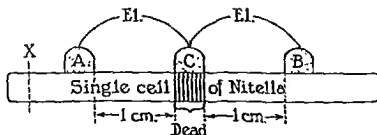


FIG. 6. Diagram to show the arrangement of an experiment. The spot *C* was killed by chloroform before the cell was stimulated: 0.001 *M* KCl was applied at all contacts.



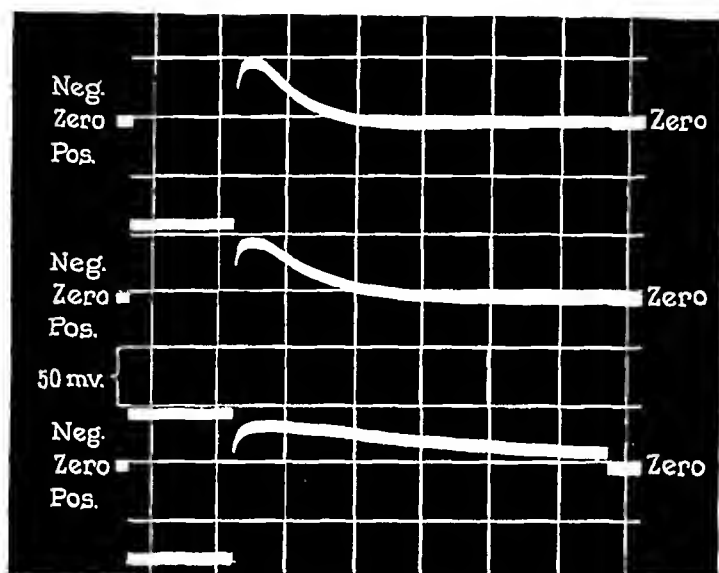


FIG. 7. Photographic record of an experiment arranged as in Fig. 8. The uppermost curve shows the P.D. of *A* with respect to *C*, the middle curve that of *B* with respect to *C*, and the lowest curve that of *D* with respect to *C*. When the cell was cut at *X* a death wave traveled down the cell, losing intensity as it progressed, as shown by the lessened amplitude and speed of the movements constituting the response.

The interval between the vertical lines is 5 seconds.

In this case the curve has but one crest but more commonly it has two as in Fig. 1*a*.

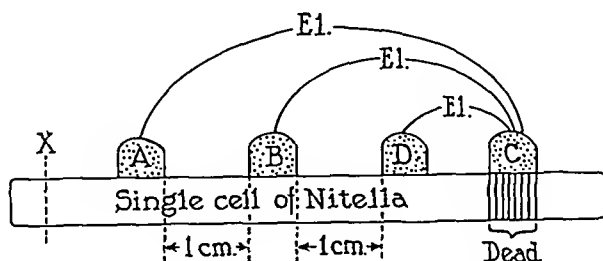


FIG. 8. Diagram to show the arrangement of an experiment. The spot *C* was killed by chloroform before the cell was stimulated: 0.001 M KCl was applied at all contacts.

movements (but not the speed of transmission<sup>4</sup>) fall off<sup>5</sup> as the stimulus moves along the cell.

On superficial examination the curve of a mechanical variation (lower curve in Fig. 5 and both curves in Fig. 10) might be confused with that of a propagated negative variation such as is shown<sup>6</sup> in Fig. 9, but there are evident differences. In the first place, the upward movement in Figs. 5 and 10 is much more abrupt (the record is

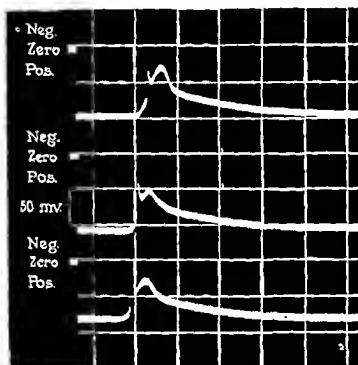


Fig. 9. Photographic record of an experiment arranged as in Fig. 8. The uppermost curve records the p.d. of *A* with reference to *C*, the middle curve that of *B* with reference to *C*, and the lowest curve that of *D* with reference to *C*. A propagated negative variation started by chemical stimulation at *C* (Fig. 8) appeared at *D* (lowest curve), then at *B* (middle curve), and a little later at *A* (uppermost curve).

The interval between the vertical lines is 5 seconds.

<sup>4</sup> The speed of transmission is estimated from the first movement *a* observed on fast records.

<sup>5</sup> The amplitude of the response also falls off as a mechanical disturbance travels in a rubber tube, as elsewhere explained.<sup>2</sup> This amplitude likewise diminishes as the force of the blow decreases.

<sup>6</sup> In this case the curves do not go to zero. This will be discussed in later papers.

too slow<sup>7</sup> to show the first downward movement *a*). In the second place the time of transmission differs; in Figs. 5 and 10 the first upward movement *b* appears to be simultaneous in the upper and lower

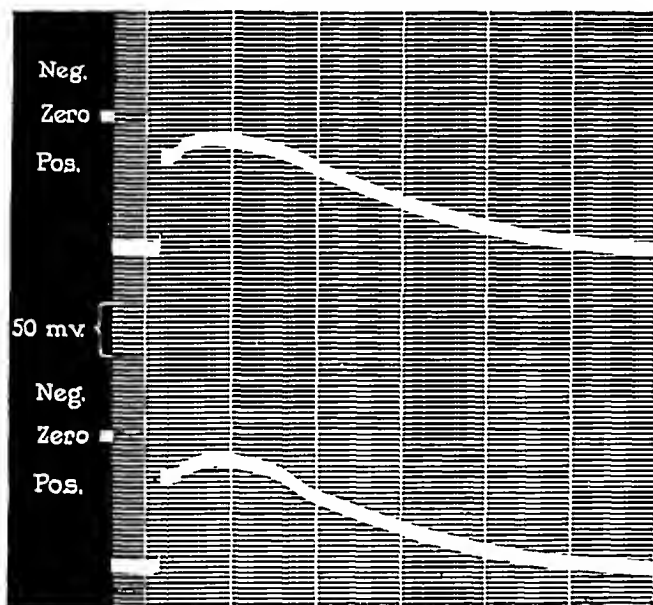


FIG. 10. Photographic record of an experiment arranged as in Fig. 3. The upper curve records the P.D. of *A* with reference to *C* and the lower curve that of *B* with reference to *C*. When the cell was pinched at *P* (Fig. 3) a mechanical disturbance traveled along the cell producing mechanical variations at *B* (lower curve) and at *A* (upper curve). The responses appear to be simultaneous in this record.

The interval between the vertical lines is 5 seconds.

curves (and presumably this applies also to the downward movement *a*), but in Fig. 9 the first upward movement<sup>7</sup> occurs later at *A* (uppermost curve) than at *B* (middle curve). The difference in time between the first upward movement<sup>8</sup> at *A* and *B* is about 1 second (and

<sup>7</sup> Figs. 1 *a*, 1 *b*, and 1 *c* are based on faster records in which the photographic paper moved at least 12.5 cm. per second.

<sup>8</sup> This need not always be the case. For the corresponding relations in movements *c* and *d* see Osterhout, W. J. V., and Hill, S. E., *J. Gen. Physiol.*, 1930-31, 14, 385.

this is also true for *B* and *D*). Since the distance between *A* and *B*<sup>9</sup> (and between *B* and *D*) is 1 cm. the rate of transmission is about 1 cm. per second.<sup>10</sup>

The transmission time is characteristic. Mechanical variations as seen<sup>6</sup> in Fig. 10 have the appearance of being simultaneous at both

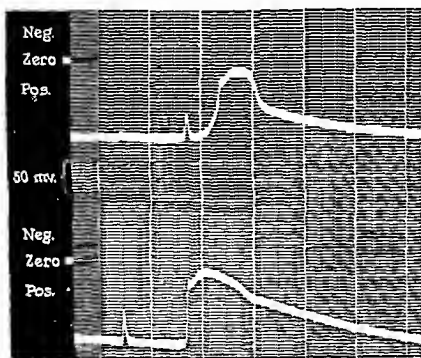


FIG. 11. Photographic record of an experiment arranged as in Fig. 3. The upper curve records the P.D. of *A* with reference to *C* and the lower curve that of *B* with reference to *C*. When the cell was pinched at *P* a mechanical disturbance traveled rapidly to *B*, producing an incomplete mechanical variation (lower curve), and then to *A*, producing a still smaller response (upper curve). A second pinch produced a complete mechanical variation at *B* and an incomplete one at *A*, but a little later a propagated negative variation arrived at *A* (it is characterized by a gradual upward movement and a slow rate of transmission).

The interval between the vertical lines is 5 seconds.

contacts: the figure indicates that the mechanical disturbance, produced by pinching at *P* (Fig. 3), traveled rapidly along the cell, pro-

<sup>9</sup> I.e., the distance from the right edge of contact *A* to the left edge of contact *B*. Cf. footnote 2.

<sup>10</sup> In this case the propagated negative variation originated by chemical stimulation at *C* as elsewhere explained (Osterhout, W. J. V., and Hill, S. E., *J. Gen. Physiol.*, 1929-30, 13, 459).

ducing the same response at *B* (lower curve) and at *A* (upper curve). The mechanical disturbance probably travels at the rate of sound in water, as elsewhere explained,<sup>2</sup> in which case the responses at *A* and *B* would appear to be simultaneous on our records.

When the intensity of the mechanical disturbance falls off so that it can no longer produce a complete mechanical variation (*i.e.*, one whose curve goes to zero), it often produces a less extensive change, which may be called an incomplete mechanical variation. Such changes are shown<sup>6</sup> in Fig. 11 resulting from a bend at *P* (Fig. 3) which produced a small response at *B* (lower curve) and a still smaller one at *A* (upper curve). A few seconds later the cell was more vigorously bent at *P* and we see at *B* a complete mechanical variation (going practically to zero) and simultaneously an incomplete one at *A*. Soon afterwards *A* made still another response whose gradual upward movement and slow rate of transmission are characteristic of a propagated negative variation. The interval between the first visible movement<sup>11</sup> at *B* and the slow upward movement at *A* after the second pinch is about 1 second and as the distance is 2 cm. we may regard the rate of transmission as about 2 cm. per second (the speed of a propagated negative variation<sup>12</sup>).

It would seem that a mechanical disturbance strong enough to produce a complete local variation at *B* fell off so much that when it reached *A* it caused only a small response and presumably nothing more would have happened at *A* had not the local variation at *B* passed over into a propagated negative variation which then traveled on to *A*. A change of this sort is to be expected if a mechanical disturbance can greatly reduce the p.d. across the protoplasm at any point and travel no further, for a flow of current will presumably start from the next point<sup>13</sup> having a sufficient p.d. and so produce a propagated negative variation.

<sup>11</sup> *I.e.*, between the start of the upward movement at *A* and that at *B*: this can be determined with sufficient accuracy for our present purpose which is merely to find the approximate rate of transmission.

<sup>12</sup> Cf. Blinks, L. R., Harris, E. S., and Osterhout, W. J. V., *Proc. Soc. Exp. Biol. and Med.*, 1928-29, 26, 836.

<sup>13</sup> Cf. Osterhout, W. J. V., and Hill, S. E., *J. Gen. Physiol.*, 1929-30, 13, 459, 547. Osterhout, W. J. V., *Electrical phenomena in the living cell*, in Harvey Lectures, 1929-30, The Williams and Wilkins Co., Baltimore, 1931, 169.

In some cases the change from a mechanical to a propagated variation may occur so early that we see little or nothing at the second contact save the propagated negative variation. This is most often observed when the bending or pinching is so gentle that the cell can be repeatedly stimulated in the same spot without seeming to suffer any permanent injury.<sup>14</sup> As an illustration we may turn<sup>6</sup> to Fig. 12. Bending at *Q* (Fig. 3) produced a mechanical variation at *A* (as

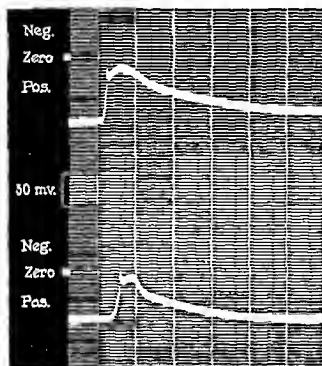


FIG. 12. Photographic record of an experiment arranged as in Fig. 3. The upper curve shows the p.d. of *A* with reference to *C* and the lower curve the p.d. of *B* with reference to *C*. When the cell was bent at *Q* a mechanical disturbance traveled to *A* producing a mechanical variation (upper curve) but the intensity of the disturbance fell off so that it changed over to a propagated negative variation, as shown by the gentle upward slope at *B* (lower curve) and slow rate of transmission.

The interval between the vertical lines is 5 seconds.

shown by the abrupt upward movement on the upper curve). But before it reached *B* it passed over into a propagated negative variation (as shown by the gentle upward slope of the first movement on the lower curve): this appeared at *B* about a second after the first move-

<sup>14</sup> A very vigorous bend or pinch may produce irreversible changes (death waves).

ment at *A*, and as the distance between *A* and *B* is 1 cm. the rate of transmission is about 1 cm. per second (*i.e.*, that of a propagated negative variation).

It seems justifiable to conclude that a mechanical disturbance may produce a death wave which, as it travels, may lose intensity so that it can set up only a mechanical variation and later only an incomplete one which may finally pass over into a propagated negative variation.

It is of interest to note that in the spring and fall cells are often found which cannot be stimulated either electrically or chemically to give propagated negative variations but repeated bending or pinching at the same spot (at intervals of 20 seconds or more) may produce each time a mechanical variation (which on superficial examination might pass for a propagated negative variation).

How does a mechanical disturbance produce an electrical response? Aside from streaming potential<sup>15</sup> a rupture of the non-aqueous surface film might lessen or abolish the P.D. across the protoplasm: possibly the mechanical disturbance may cause ions to move into the protoplasm and thus lower the P.D. without rupturing the film.<sup>16</sup> As such changes could not arise in a homogeneous medium they furnish evidence in favor of the idea that the aqueous protoplasm is covered by non-aqueous films.

It would be interesting to know how general such phenomena are in the field of mechanical stimulation, which includes such diverse features as the senses of touch and hearing, the mechanical stimulation of motile organs in plants,<sup>17</sup> and thigmotropism; geotropism seems to be in the same category as it depends on deformation of the protoplasm.<sup>18</sup>

The all or none law does not apply to incomplete mechanical variations, for the response varies with the strength of the stimulus. Although this is sufficiently evident from a qualitative standpoint it

<sup>15</sup> The effects of streaming potential (which have been considered in a previous paper<sup>2</sup>) seem to be relatively transitory.

<sup>16</sup> Osterhout, W. J. V., *Electrical phenomena in the living cell*, in *Harvey Lectures*, 1929-30, The Williams and Wilkins Co., Baltimore, 1931, 169. Osterhout, W. J. V., and Hill, S. E., *J. Gen. Physiol.*, 1929-30, 13, 547. Blinks, L. R., *J. Gen. Physiol.*, 1929-30, 13, 361, 495.

<sup>17</sup> Cf. Stern, K., *Elektrophysiologie der Pflanzen*, Julius Springer, Berlin, 1924.

<sup>18</sup> This might occur either with or without otoliths, starch grains, etc.

cannot be investigated quantitatively until we have a satisfactory method of regulating the strength of the stimulus.<sup>19</sup>

There is an interesting point touching the form of the curves. Gasser and Erlanger<sup>20</sup> have shown that in the case of nerve the double crest indicates different rates of conduction in different fibres. Evidently the double crest in our curves cannot be explained by assuming different rates of conduction in different parts of the protoplasm. For if this were the case we should have a wider separation of the crests as the conducting path lengthened but this is certainly not the rule and occasionally we find records like Fig. 9 which shows that the contact nearest to the point of stimulation (lowest curve) has a greater separation of crests than the others. Furthermore we find (Figs. 5, 10, 11, and 12) a double crest where no conduction of the stimulus is supposed to occur but each variation is due to a mechanical stimulus acting at the point where the response occurs.

We desire to add that we began our investigation by repeating preliminary experiments made by Mr. E. S. Harris<sup>21</sup> (in collaboration with the senior author).

#### SUMMARY

Mechanical stimulation of *Nitella* often produces responses resembling propagated negative variations but traveling faster and going past a killed spot. They appear to result from a mechanical disturbance traveling along the cell and stimulating each spot it touches (*i.e.* the stimulus itself travels). They are called mechanical variations to distinguish them from propagated negative variations.

A mechanical disturbance may cause an irreversible change (death wave), but in traveling along the cell it may lose intensity and then produce only a reversible response (mechanical variation) which may eventually change to a propagated negative variation.

The all or none law does not apply to incomplete mechanical variations, for the response varies with the strength of the stimulus.

<sup>19</sup> It is easy to strike the cell a measured blow, but since the effect depends on the physical characteristics of the cell (*e.g.*, diameter, flexibility of wall, turgidity, etc.) a given series of blows can be compared only qualitatively from cell to cell.

<sup>20</sup> Gasser, H. S., and Erlanger, J., *Am. J. Physiol.*, 1927, 80, 522.

<sup>21</sup> Blinks, L. R., Harris, E. S., and Osterhout, W. I. V., *Proc. Soc. Exp. Biol. and Med.*, 1928-29, 26, 836.





# A METHOD FOR THE RAPID DIALYSIS OF LARGE VOLUMES OF PROTEIN SOLUTIONS

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(Accepted for publication, January 30, 1931)

The removal of salts from solutions of proteins can be a laborious task. The method of Kunitz and Simms (1) was an important step in the direction of making this task simpler. We have found it convenient and necessary to be able to dialyze proteins rapidly, without undue loss due to denaturing, and without using a preservative. Methods of dialysis without circulation of the protein solution and of the outside liquid take several days, and frequently weeks, necessitating the employment of preservatives. Spontaneous denaturing of the protein is appreciable during long periods of dialysis. The method here described largely avoids these difficulties. It can be varied in certain ways. For example, the outside air can be excluded; the angle of the membrane-support can be changed; greater or less economy of distilled water can be effected. The whole apparatus can be placed in a cold room, or other methods can be employed to keep the temperature low.

## *Description of Apparatus*

The design of the apparatus is indicated in Figs. 1-3. The outside liquid is contained in an ordinary metal wash basin (A), paraffined on the inside, fitted with intake pipe (B) and overflows (C and C') to permit the circulation of water as indicated in Fig. 1. The cover of this vessel (D), on which a constant speed motor (E) is mounted, is just wide enough to support the motor and the casing for the drive shaft (F), which is inclined at an angle of approximately 70° to the horizontal. The supporting racks (G), of which one or two may be attached to the shaft by a sleeve and screw (H), can each support six membranes. The method of supporting the membranes on the rack is shown in Figs. 2 and 3. The membranes (I) are held in place by the pegs (K) on the rack, by two long rubber bands (L and L') stretched completely across the pegs and passing over the membranes, and by glass rods (M) which pass through single-hole rubber stoppers fixed in the mem-

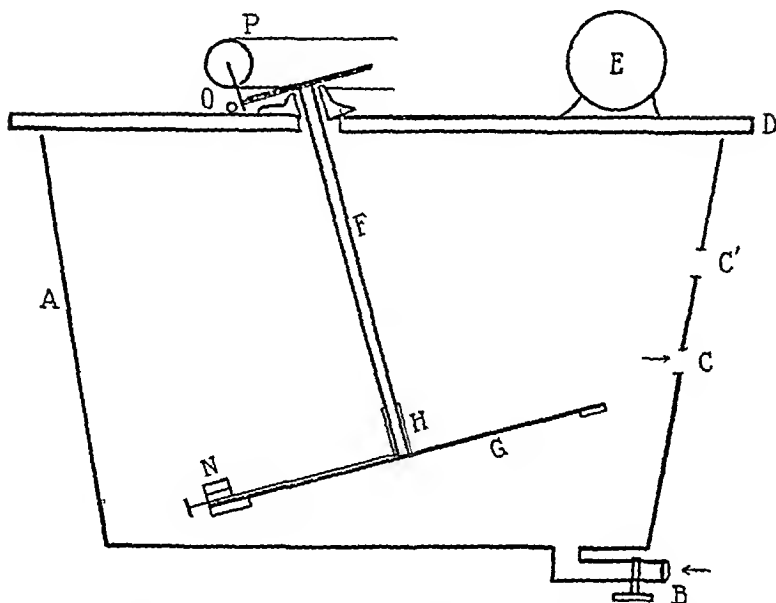


FIG. 1. Cross-section through entire apparatus.  $\times 1/6$  approximately. (See text.)

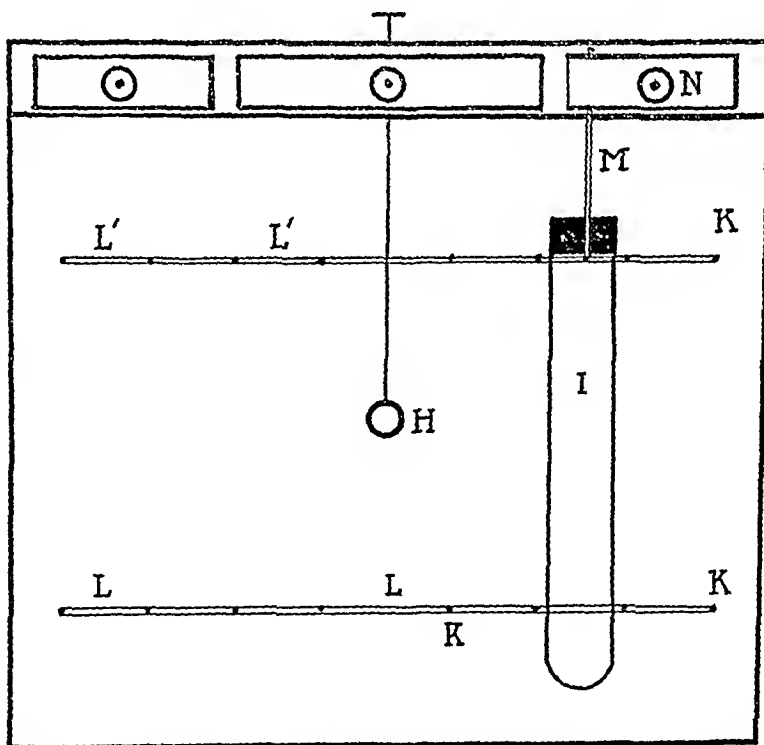


FIG. 2. Top view of rack (G) that supports membranes.  $\times 3/10$  approximately. (See text.)

branes. These rods in turn are fixed in place by the spring clamps (*N*) on the rack. The drive shaft is connected with the motor by the worm gear (*O*) and pulley (*P*).

At first the membranes were prepared by depositing in the usual fashion three layers of collodion, U.S.P.X, on the inside of  $27 \times 200$  mm. test tubes, and drying in air for 20 minutes. Later this type of membranes was supplemented by the use of 25 mm. diameter "Visking" tubes,<sup>1</sup> which proved more satisfactory in that the necessity for the preparation of collodion membranes was eliminated. Using this apparatus and the "Visking" tubes, twelve membranes, each containing 100 cc. of crystals or of solution, may be dialyzed simultaneously.

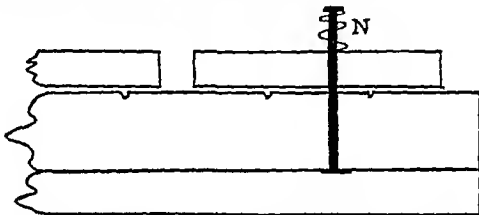


Fig. 3. Schema of arrangement for attachment of membrane.  $\times 2/3$

#### EXPERIMENTAL

The cylindrical membranes are filled to within a few cm. of the top with the solution to be dialyzed and are fixed in place on the rack. Tap water is then allowed to flow through the outer vessel at a rate of 1.5 to 4 liters per minute. As the inclined rack is rotated at 6 R.P.M. by the motor, the air bubble<sup>2</sup> in the membrane tube provides gentle stirring. When dialysis against tap water is complete the outer vessel is drained, the intake pipe shut off, and dialysis is continued against distilled water. In obtaining the illustrative data given in Table I only dialysis with one change of distilled water was used.

It was found that denaturing of protein was negligible during the short time required for dialysis. The loss of protein by leakage was

<sup>1</sup> Kindly supplied by the Visking Corporation. Two stoppers, one of them single-holed, are fixed with rubber bands into the ends of the Visking tubing. It is necessary to test each membrane for leaks.

<sup>2</sup> We are indebted to Professor Crozier for the suggestion that a smooth glass sphere may be more suitable than the air bubble since probably less denaturation would occur.

determined by the Koch-McMeekin method, and was found to amount to less than 5 per cent after 12 hours' dialysis. The rate of disappearance of salt was determined both with pure salt solutions and with solutions containing proteins. Half-saturated ammonium sulfate was dialyzed ammonia-free (as tested with Nessler-Folin reagent) within 12 hours. Some of the results obtained with the protein solutions are given in Table I. Not more than three membranes were employed in these experiments. The rate of dialysis against distilled water may be appreciably affected by the number of membranes used in each experiment.

TABLE I

These data illustrate the rate of disappearance of salts from cylinders of collodion and of "Visking" tubing under the experimental conditions described in the text.

| Protein solution                           | Time dialyzed (hours) |           | Spec. conductivity<br>$\times 10^5$ reciprocal<br>ohms |
|--|-----------------------|-----------|--|
|  | Tap water             | Distilled |  |
| 1. Serum globulin                          | 24                    | 0         | 13.8   |
| 2. Egg albumin (10 per cent)               | 12                    | 12        | 16.9   |
| 3. Egg albumin (2 per cent, approximately) | 2½                    | 0         | 857.0  |
|  | 5½                    | 0         | 96.3   |
|  | 7¼                    | 0         | 42.0   |
|  | 8                     | 0         | 36.9   |
|  | 8                     | 4         | 13.9   |

The conductivity of the tap water used was  $12.0 \times 10^{-5}$ ; the conductivity of the distilled water (after exposure to the air) was  $1.40 \times 10^{-5}$  reciprocal ohms. In the dialysis of Solutions 1 and 2, collodion sacks were used. In the dialysis of Solution 3, "Visking" membrane was used. In all cases the solutions were half saturated in ammonium sulfate before dialysis.

These results, especially those obtained with Solution 3, compare favorably with the value of  $18 \times 10^{-5}$  obtained by Kunitz and Simms (1) after 24 hours dialysis against circulating distilled water, and the value of  $16 \times 10^{-5}$  obtained by them after 31 hours.<sup>3</sup>

<sup>3</sup> The apparatus can be obtained from Mr. J. H. Emerson of Cambridge, Mass.

## SUMMARY

A method is described by which moderately large quantities of protein solutions can be dialyzed relatively free of salts within 12 to 24 hours. The use of "Visking" tubes instead of collodion bags is recommended, for speed of dialysis as well as ease of manipulation.

## CITATION

1. Kunitz, M., and Simms, H. S., *J. Gen. Physiol.*, 1928, 11, 641.



# THE SORPTION OF BACTERIOPHAGE BY LIVING AND DEAD SUSCEPTIBLE BACTERIA

## I. EQUILIBRIUM CONDITIONS

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(Accepted for publication, January 20, 1931)

The first step in the process of bacteriophagy, or lysis of bacteria by bacteriophage, is the combination of phage with susceptible organisms. D'Herelle in his early work (1, 2) noted a rapid and specific attachment of phage to susceptible bacteria under suitable conditions, and von Angerer (3), working with dead organisms, found that the process could be expressed in the form of the usual adsorption isotherm equation with  $1/n = 0.75 - 0.80$ . Prausnitz and Firle (4) further observed that heating susceptible bacteria to temperatures above  $110^{\circ}\text{C}$ . resulted in destruction of the ability to bind phage. It is also known that as a general rule homologous or heterologous resistant bacteria fail to combine with phage to any appreciable extent (4, 5, 6); although Flu (7) and Gobs and Jacobsohn (8) have reported cases which are definite exceptions to the rule.

The general trend of the above work has led to the assumption that live bacteria take up phage by adsorption, although there exists no adequate quantitative data in support of this idea. Obviously the question of how phage and bacteria combine is a fundamental one and the writer has felt that a closer study of the process would be in order, particularly since the observation of Krueger and Northrop (9) that extracellular phage per milliliter constitutes a constant low percentage of total phage per milliliter during the logarithmic phase of phage production in a phage-bacteria mixture, suggests the possibility of phage distribution being of a simple type.

The present paper deals with observations on the distribution of phage between bacteria (both living and dead) and the broth in which



they are suspended. The phage used throughout is a highly active antistaphylococcus race and the bacterium is a single phage-susceptible strain of *S. aureus*. Abbreviations and terms employed are defined as follows:

*P*: Bacteriophage.

*B*: Bacterium.

[*P*]: Phage/ml.

[*B*]: Bacteria/ml.

P.U.: Phage unit. An arbitrary activity unit (11).

[*P*]<sub>e</sub>: Unbound phage/ml. at equilibrium.

*P*<sub>b</sub>: Phage bound by bacteria.

Broth: Veal infusion, 1 per cent peptone, 0.5 per cent NaCl, pH 7.6.

*Distribution of Phage between Living, Susceptible Bacteria and Broth in the Absence of Growth*

*Methods*: 16 hour cultures of *S. aureus* grown in Blake flasks were taken up in saline, filtered through a Schleicher and Schüll Faltenfilter No. 588, washed twice in saline and the cell content determined by the centrifuged-sediment method (10). The saline suspension was diluted with broth to give a proper [*B*] and the *B*-broth mixture was held at 10°C. for  $\frac{1}{2}$  hour before being used. Mixtures of *P* and *B* (already cooled to 10°C.) were placed in test tubes and the latter were shaken at 10°C. for 2 hours. At the end of this time the tubes were placed in iced holders and were centrifuged at high speed for 6 minutes. The supernatants were at once pipetted off, diluted with broth, and kept at 0°C. until they could be titrated.

For determining free *P* at equilibrium 4 ml. aliquots of various dilutions were employed, the titration procedure being that described by Krueger (11, 12). Phage combined with *B* was determined by difference. Controls of *B* in broth without *P* and of known [*P*]'s mixed with *B* (diluted for titration without centrifuging, for total *P*/ml.) were run with each series to detect any bacterial reproduction or formation of *P*.

Since the relationship being studied was that existing between *P* and *B* in the absence of *B* growth, it was essential to prevent bacterial reproduction. Rather than complicate the experiments by introducing chemical inhibitors, it seemed best to work at low temperatures. It soon developed that an additional factor of safety was introduced if the broth suspension of *B* was kept at 10°C. for  $\frac{1}{2}$  hour before being mixed with *P*. The controls of *B* alone in broth were sufficient to detect cases of moderate *B* growth, but a much more satisfactory growth criterion lay in the control mixtures of known [*P*]'s with *B*.

The value of such controls rests upon a fact elicited in previous work on the kinetics of the *B-P* reaction (9), namely, that the rate of *P* production in a *P-B* mixture is a power of the rate of *B* reproduction. Consequently, even slight increases in  $[B]$  give rise to very appre-

TABLE I

*Distribution of Phage Between Broth and Live, Resting Bacteria at 10°C.  
Volume 4 Ml. Period of Contact 2 Hours*

| Total initial <i>P</i> , u.  | Total initial <i>B</i> | $[P]_e$                                   | $K = \frac{(P_b/B)(25 \times 10^{10})}{[P]_e}$ |
|--|------------------------|---|--|
| $4 \times 10^3$  | $16 \times 10^3$       | $5 \times 10^7$                           | $11.9 \times 10^3$                             |
| 2 "  | "                      | 3 "                                       | 9.8 "  |
| 1 "  | "                      | 1.6 "                                     | 9.2 "  |
| $8 \times 10^3$  | "                      | $9.5 \times 10^6$                         | 12.5 "   |
| 4 "  | "                      | 5.0 "                                     | 11.9 "   |
| 2 "  | "                      | 2.75 "                                    | 10.7 "   |
| $4 \times 10^3$  | $8 \times 10^3$        | $1.02 \times 10^8$                        | 11.0 "   |
| 2 "  | "                      | $5.0 \times 10^7$                         | 11.2 "   |
| 1 "  | "                      | 2.3 "                                     | 12.3 "   |
| $8 \times 10^3$  | "                      | 1.95 "                                    | 11.6 "   |
| 4 "  | "                      | 1 "                                       | 11.2 "   |
| 2 "  | "                      | $5.75 \times 10^6$                        | 9.6 "  |
| $4 \times 10^3$  | $4 \times 10^3$        | $2.1 \times 10^8$                         | 9.4 "  |
| 2 "  | "                      | $9.75 \times 10^7$                        | 10.3 "   |
| 1 "  | "                      | 5 "                                       | 10.0 "   |
| $8 \times 10^3$  | "                      | 3.8 "                                     | 10.8 "   |
| 4 "  | "                      | 1.75 "                                    | 11.8 "   |
| 2 "  | "                      | $8.0 \times 10^6$                         | 13.1 "   |
| Control for <i>B</i> growth and<br><i>P</i> formation<br>$4 \times 10^3$             | $16 \times 10^3$       | Final total <i>P</i> : $3.97 \times 10^3$ |  |
| Control for <i>B</i> growth (Cen-<br>trifuged-sediment<br>method)<br>$4 \times 10^3$ | $16 \times 10^3$       | Final total <i>B</i> : $4.07 \times 10^3$ |  |

ciable and easily detectable increments in total  $[P]$ . *P* production therefore serves as a sensitive indicator of *B* growth.

Control tests run at intervals of 15 minutes on several distribution series indicated that  $1\frac{1}{2}$  hours sufficed for the establishment of equilibrium between intracellular and extracellular *P*. This point

was also confirmed by experiments in which equilibrium was approached from both sides (see reversal experiments). The use of periods longer than 3 to 4 hours for contact between *P* and live, non-growing *B* introduced complications due to the death of considerable numbers of *B*. It is shown elsewhere (Distribution of phage between heat-killed susceptible bacteria and broth) that the processes by which dead *B* and live *B* take up *P* from solution differ. Also, control experiments indicate that under the experimental conditions obtaining no significant drop in viable survivors occurs during the first  $3\frac{1}{2}$  to 4 hours. After this time, however, the proportion $\frac{1}{2}$  of dead

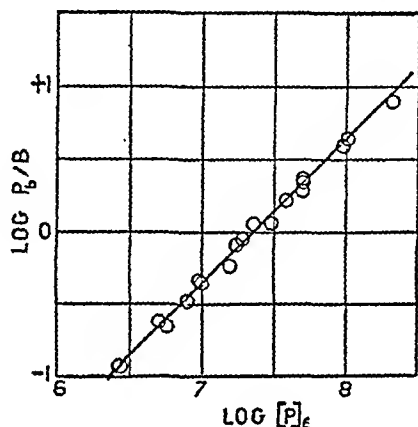


FIG. 1. Phage distribution between live, resting, susceptible bacteria and broth. Data plotted as adsorption isotherm. Contact period 2 hours at  $10^{\circ}\text{C}$ . The points are seen to lie along a line of slope  $(1/n) = 1$ .

cells in the mixture becomes considerable and their presence accounts for the inconstant and not altogether predictable results secured when long contact periods are employed.

Table I lists the pertinent data obtained in one distribution experiment run at  $10^{\circ}\text{C}$ .  $[P]$ 's varied from  $1 \times 10^9$  p.u./ml. to  $5 \times 10^7$  p.u./ml. and over this range three different  $[B]$ 's were employed:  $4 \times 10^8$ /ml.,  $2 \times 10^8$ /ml. and  $1 \times 10^8$ /ml. respectively. A period of 2 hours was allowed for the system to come to equilibrium before samples were taken, and it is apparent that the observed values give a reasonably constant partition coefficient. In calculating  $K$  the ratio  $P_b/B$  is multiplied by  $25 \times 10^{10}$  in order to obtain the concentration

of  $P$ /ml. of  $B$  (there are approximately  $25 \times 10^{10}$  bacterial cells in 1 ml. of packed bacteria). Fig. 1 presents the same data plotted in the log form of the adsorption isotherm equation. The points are seen to lie on the line of slope 1 (*i.e.*,  $1/n = 1$ ).

Any single  $B$  suspension gives satisfactorily constant values for the partition coefficient. Different preparations of  $B$ , however, produce moderate fluctuations in the coefficient over and above the variations due to experimental errors in estimating  $B$  and determining  $P$ . [Determinations of  $B$  can be made with an accuracy of  $\pm 2$  per cent (10) while  $[P]$  can be determined within  $\pm 3$  per cent (12).]

It would appear then that the distribution of  $P$  between broth and live, non-growing  $B$  may be expressed in the form of the common Normal Distribution Equation. If the distribution is strictly normal it not only should furnish data which fit the equation but equilibrium should be attainable from both sides. Experimentally this proves to be true as is demonstrated in the following experiment:

$4 \times 10^9$  P.U. and  $1.6 \times 10^9$   $B$  were mixed in a total volume of 4 ml. of broth. The mixture was shaken for  $1\frac{1}{2}$  hours at  $10^\circ\text{C}$ . to permit establishment of equilibrium and then was diluted 1/10 with broth. After an additional  $1\frac{1}{2}$  hours shaking at  $10^\circ\text{C}$ .  $[P]_e$  of the diluted mixture was determined. For comparison  $[P]_e$  was also ascertained in mixtures having the  $B$  and  $P$  concentrations of the undiluted and diluted original set-up. For this purpose two tubes were prepared: in both of which  $[P] = 1 \times 10^9$  and  $[B] = 4 \times 10^8$ . To be sure that equilibrium had been reached free  $P$ /ml. was determined in one tube at  $1\frac{1}{2}$  hours and in the other at 2 hours. To check  $[P]_e$  in the diluted suspension a flask containing  $4 \times 10^9$  P.U. and  $1.6 \times 10^9$   $B$  in 40 ml. of broth was included. Samples for determinations of free  $P$ /ml. were taken at  $1\frac{1}{2}$  and 2 hours.

As shown in Table II the partition coefficients agree quite well. Moreover, the total free  $P$  after dilution is nearly ten times the amount present before dilution, so there is no doubt that equilibrium in the dilute suspension was reached by diffusion of phage from the bacteria instead of diffusion into them, as is the case in the previous experiments. Consequently it follows that the removal of  $P$  from solution into living, susceptible  $B$  is a reversible process, thus further substantiating the idea that  $P$  distribution between  $B$  and broth in the absence of growth is a case of true Normal Distribution. Repetition of this experiment at  $0^\circ\text{C}$ . and  $10^\circ\text{C}$ . with other concentrations of  $B$  and  $P$  has given like results.



*Distribution of Phage Between Growing Susceptible Bacteria and Broth*

In an earlier paper (9) Krueger and Northrop reported a simple relationship found to exist between intracellular and extracellular  $P$  in a  $P$ - $B$  mixture during the log phases of  $B$  growth and  $P$  formation. The log plots of total  $P$ /ml. and extracellular  $P$ /ml. against time in several of our kinetic experiments were practically parallel lines, indicating that extracellular  $P$ /ml. was a constant low percentage (*ca.* twenty per cent) of total  $P$ /ml., while the intracellular  $P$  fraction constituted a constant major percentage of total  $P$ /ml. at any particular time.

TABLE III

*Calculation of Partition Coefficients for Phage Distribution in Phage-Growing B Mixture at 36°C. Data from a Previously Reported Experiment (9)*

| $t$ (sampling) | $B$ /ml           | Total $P$ /ml.       | Free $P$ /ml.     | $K = \frac{(P_b/B)(25 \times 10^{10})}{[P]_{free}}$ |
|----------------|-------------------|----------------------|-------------------|---|
| <i>Hrs.</i>    |                   |                      |                   |   |
| 0              | $2.5 \times 10^7$ | $2.5 \times 10^4$    | $2.5 \times 10^4$ | —   |
| .50            | 4 "               | $2.5 \times 10^5$    | 1.6 "             | $90 \times 10^3$                                    |
| 1.00           | 6.3 "             | $2 \times 10^6$      | 6.3 "             | 123 "   |
| 1.50           | $1.6 \times 10^8$ | $2 \times 10^7$      | $4 \times 10^5$   | 76 "  |
| 2.00           | 3.2 "             | $1.6 \times 10^8$    | $4 \times 10^5$   | 31 "  |
| 2.50           | 6.3 "             | $1.6 \times 10^8$    | $4 \times 10^7$   | 16 "  |
| 3.00           | $1 \times 10^9$   | $1.6 \times 10^{10}$ | $5 \times 10^8$   | 7.7 "   |
| 3.50           | "                 | $1.4 \times 10^{11}$ | $1.6 \times 10^9$ | 21.6 "  |

With  $[B]$  constant this relationship would signify that a true normal distribution obtained with reference to  $P$  in the bacterial and broth phases. However, the system is not static, for  $[B]$  is increasing logarithmically with time and *a priori* the data will not yield a constant partition coefficient. Table III, an analysis of such an experiment, shows this to be true,  $K$  decreasing markedly during the period studied.

Since the distribution of  $P$  between living susceptible  $B$  and broth in the absence of growth is so definitely of normal type it seemed advisable to reconsider our earlier kinetic data. In the first place, there was the possibility that with such a rapid rate of  $P$  production there might exist a lag in diffusion of  $P$  through the cell membrane,

samples being taken too fast for attainment of equilibrium. To influence the data in the direction of maintaining a constant partition coefficient we should have to assume then that  $P$  is formed extracellularly and that a progressively greater lag arose in the diffusion of  $P$  into the cell. While there was no special reason to suppose that this represented the true situation the possibility could be experimentally checked. The test was carried out by allowing a  $P$ -growing  $B$  mixture to reach a point well within the log phase of  $B$  growth, stopping the reaction by rapidly cooling the reactants to  $0^\circ\text{C}$ . and titrating the free and total  $P/\text{ml}$ . at intervals. Table IV gives the

TABLE IV

*Experiment to Detect Lag in  $P$  Distribution Between Cell and Broth*  
 $[B]_0$   $2.5 \times 10^7$   $[P]_0$   $1 \times 10^7$  Volume 30 ml.  
*Mixture Shaken at  $36^\circ\text{C}$ . 1 3/4 Hours. Cooled at Once to  $0^\circ\text{C}$ . Samples Taken as Indicated*

| $t$ (after icing) | $B/\text{ml}$ .   | Total $P/\text{ml}$ . | Free $P/\text{ml}$ . | $K = \frac{(P_b/B)(25 \times 10^{10})}{[P] \text{ free}}$ |
|-------------------|-------------------|-----------------------|----------------------|---|
| <i>min.</i>       |                   |                       |                      |   |
| 1                 | $1.8 \times 10^8$ | $5.70 \times 10^8$    | $3 \times 10^8$      | $25 \times 10^8$  |
| 15                | "                 | 5.60 "                | "                    | 24.5 "  |
| 30                | "                 | 5.65 "                | $3.1 \times 10^8$    | 24.0 "  |
| 45                | "                 | 5.70 "                | "                    | 24.2 "  |

essential data of the experiment and shows that no lag in diffusion of  $P$  either in or out of the cell is detectable.

The next desirable bit of information was the exact relative slopes of the log plots of extracellular  $P/\text{ml}$ . and intracellular  $P/\text{ml}$ . against  $t$ . In the experiments previously reported (9) samples taken for total  $P/\text{ml}$ . determinations were diluted at once and were mixed with the test  $B$  suspension. The time period occupied by the procedure was short and the titration data furnished very uniform slopes for the rate of total  $P$  formation. However, this was not so in the case of free  $P/\text{ml}$ . determinations. These samples had to be centrifuged for 0.1 hour during which time the  $P$ - $B$  reaction continued. They were then diluted and titrated immediately.

Plots of the titration readings, *i.e.*, log extracellular  $P/\text{ml}$ . against

*t*, gave slopes varying somewhat in different experiments and the points of any one plot were not as regularly aligned as in the total *P*/ml. plots. To obtain better data the same experiments were repeated with this alteration in technic: Samples for total *P*/ml. and for extracellular *P*/ml. were removed from the reaction mixture at intervals, chilled at once to 0°C. and the total *P* aliquots were

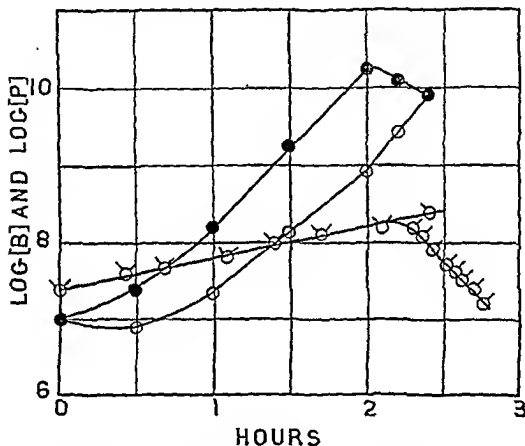


FIG. 2. Relationship of extracellular *P*/ml. to intracellular *P*/ml. during *B* growth in a *P*-*B* mixture. Experiment run at 36°C. Special sampling precautions were taken (see text) and the data from the plotted curves give a constant partition coefficient for *P* distribution between growing *B* and broth (cf. Table V). ● Total *P*/ml. ○ Extracellular *P*/ml. ▽ [*B*] in broth control. ⊗ [*B*] in *P*-*B* mixture.

diluted in 0°C. broth. Meanwhile, the chilled extracellular *P* samples were packed in ice, centrifuged 0.1 hour and the supernatants then diluted with chilled broth. All samples were held at 0°C. after dilution until the end of the experiment and were then titrated together. Control experiments showed that immediate reduction in temperature could be relied upon to stop *B* growth and *P* production and that the



Following this procedure,  $t$  of sampling, particularly for extracellular  $P$ , could be more accurately fixed and the data so obtained furnished curves of the type shown in Fig. 2. In the plot of log extracellular  $P$ /ml. against  $t$  the slope is clearly not parallel to the log Tot.  $P$ /ml. plot. The difference is slight but definite, and makes for considerable improvement in calculations of the partition coefficient. Thus in Table V the data from the experiment of Fig. 2 are listed and it is evident that the partition coefficients agree very well.

It seems reasonable to conclude therefore, that our earlier data for extracellular  $P$ /ml. were not entirely accurate due to the mechanical difficulty of fixing  $t_{(\text{sampling})}$  in a rapidly reacting mixture. With an

TABLE V

*Distribution of Phage Between Growing Susceptible Bacteria and Broth at 36°C.  
Reaction Stopped in Samples by Reduction of Temperature to 0°C.*

| $t_{(\text{sampling})}$ | $B$ /ml.          | Total $P$ /ml.    | Free $P$ /ml.      | $K = \frac{(P_b/B)(25 \times 10^{10})}{[P] \text{ free}}$ |
|-------------------------|-------------------|-------------------|--------------------|---|
| <i>hrs.</i>             |                   |                   |                    |   |
| 0                       | $3 \times 10^7$   | $1 \times 10^7$   | $1 \times 10^7$    | —   |
| .50                     | 4 "               | 2.5 "             | $7.9 \times 10^6$  | $13.0 \times 10^3$  |
| .80                     | 5.36 "            | 6.6 "             | $1.25 \times 10^7$ | 20.0 "  |
| 1.00                    | 6.3 "             | $1.6 \times 10^8$ | 2.2 "              | 25.0 "  |
| 1.20                    | 7.9 "             | 4 "               | 4.8 "              | 23.2 "  |
| 1.40                    | $1.0 \times 10^8$ | $1.1 \times 10^8$ | $1.0 \times 10^8$  | 25.0 "  |
| 1.60                    | 1.2 "             | 2.75 "            | 2.0 "              | 26.5 "  |
| 1.80                    | 1.45 "            | 7.1 "             | 4.0 "              | 29.0 "  |

improved technic, this source of error has been eliminated and the data fit the Normal Distribution Equation satisfactorily.

The distribution of  $P$  between broth and live, susceptible, growing  $B$ , i.e., in a reacting mixture of  $P$  and  $B$ , is then of the same simple type found to hold for  $P$  distribution in the case of live, non-growing  $B$ .

*Distribution of Phage between Heat-Killed Susceptible Bacteria and Broth*

*Methods.*—Saline suspensions of staphylococci were prepared as described under *Distribution of phage between living bacteria and broth in the absence of growth.* After heating at 80°C. for 3 hours the suspensions were washed several times with

saline and samples for sterility tests were taken. Various concentrations of  $P$  and  $B$  in broth were placed in the mechanical shaker at  $0^{\circ}$ ,  $10^{\circ}$  or  $36^{\circ}\text{C}$ . At the end of 16 to 18 hours the tubes were centrifuged at high speed and the  $[P]$ 's of the supernatants determined. Controls of known  $[P]$ 's with known  $[B]$ 's (for total  $[P]$  determinations) were included in each set-up. The long period of contact between  $B$  and  $P$  in these experiments was used merely as a matter of convenience and not because the attainment of equilibrium conditions required such prolonged interaction (see section on velocity, page 508).

The adsorption isotherm equation states that  $a = k c^{1/n}$ , where  $a$  = quantity of sorbate per unit of adsorbent—here p.u. adsorbed per  $B$ ,  $c$  = concentration of sorbate at equilibrium and  $k$  and  $1/n$  are

TABLE VI

*Adsorption of Phage by Heat-Killed Susceptible Bacteria at  $10^{\circ}\text{C}$ .*

*Total B:  $2.4 \times 10^8$ . Volume 8 Ml. Period of Contact 16 Hours. B Killed at  $80^{\circ}\text{C}$ .*

| Total initial p.u. | $[P]_e$            | Log $a$ (observed) | Log $a$ (calculated) from<br>$a = k c^{1/n}$ with $1/n =$<br>0.84 and $\log k = -4.25$ |
|--------------------|--------------------|--------------------|--|
| $1 \times 10^{10}$ | $1.25 \times 10^7$ | 1.66               | 1.72   |
| $4 \times 10^7$    | $3.16 \times 10^6$ | 1.22               | 1.21   |
| 2 "                | 1.15 "             | 0.92               | 0.85   |
| 1 "                | $5.0 \times 10^5$  | 0.62               | 0.55   |
| $4 \times 10^4$    | 1.86 "             | 0.22               | 0.18   |
| 2 "                | $8.1 \times 10^4$  | -0.08              | -0.12  |
| 1 "                | 4.0 "              | -0.38              | -0.39  |

constants. Table VI presents the data from an experiment typical of many performed with dead  $B$ . The constants  $\log k$  and  $1/n$  are calculated from the experimental observations and are seen to give values for  $\log a$  when substituted in the logarithmic form of the adsorption equation ( $\log a = \log k + 1/n \log c$ ) which agree well with the observed figures. In Fig. 3 the same data are plotted for comparison with the calculated curve.

Table VII includes the data from two other adsorption experiments run at  $10^{\circ}\text{C}$ . with different  $B$  preparations. In the plot of the data, Fig. 4, observed points of Fig. 2 are reproduced to bring out the degree of variation between results obtained with different lots of organisms. The two sets of data in Table VII include regions of high  $P/B$  ratios and show a distinct flattening in their upper segments due to saturation.

tion of  $B$  with  $P$ . Such behavior is not predicted by the Freundlich equation although frequently observed experimentally. However, the general adsorption theory developed by Langmuir (13) predicts this

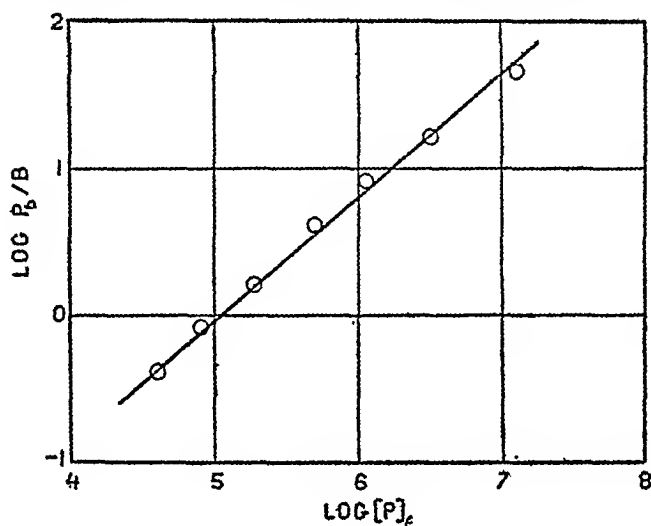


FIG. 3. Phage distribution between heat-killed, susceptible bacteria and broth. Data plotted as adsorption isotherm. Contact period 16 hours at  $10^{\circ}\text{C}$ .  $1/n = 0.84$ .

TABLE VII

*Adsorption of Phage by Heat-Killed Susceptible Bacteria at  $10^{\circ}\text{C}$ .  
Two Different B Preparations. Total B:  $2.4 \times 10^8$ . Volume 8 ML. Period of  
Contact 16 Hours. B Killed at  $80^{\circ}\text{C}$ .*

| B preparation No. 1 |                     |                     | B preparation No. 2 |                     |                     |
|---------------------|---------------------|---------------------|---------------------|---------------------|---------------------|
| Total initial p.v.  | $\text{Log } [P]_e$ | $\text{Log } P_b/B$ | Total initial p.v.  | $\text{Log } [P]_e$ | $\text{Log } P_b/B$ |
| $6 \times 10^{10}$  | 9.4                 | 2.22                | $7 \times 10^{10}$  | 9.52                | 2.26                |
| 4 "                 | 9.0                 | 2.12                | 5 "                 | 9.06                | 2.23                |
| 2 "                 | 7.9                 | 1.91                | 3 "                 | 8.35                | 2.07                |
| 1 "                 | 7.0                 | 1.61                | $8 \times 10^7$     | 6.95                | 1.52                |
| $4 \times 10^7$     | 6.40                | 1.22                | $4 \times 10^9$     | 6.60                | 1.22                |
| 2 "                 | 6.15                | 0.92                | 2 "                 | 6.24                | 0.92                |
| 1 "                 | 5.70                | 0.62                | 1 "                 | 5.80                | 0.62                |
| $4 \times 10^8$     | 5.18                | 0.21                | $4 \times 10^8$     | 5.24                | 0.22                |
| 2 "                 | 4.80                | -0.08               | 2 "                 | 4.90                | -0.08               |
| 1 "                 | 4.42                | -0.39               | 1 "                 | 4.56                | -0.38               |

type of curve with high ratios of sorbate to adsorbent and lends to the present data adequate theoretical support.<sup>1</sup>

It should be mentioned that several early adsorption experiments run at 10°C. as already described gave isotherms for which  $1/n$  in the log plot = 1.25. This suggested the possibility of  $P$  distribution in dead  $B$  being of the associative type. However, in associative distribution  $m$ , in the equation  $C_2 = k C_1^m$ , is ordinarily a whole number greater than one. Further, associative distribution is commonly

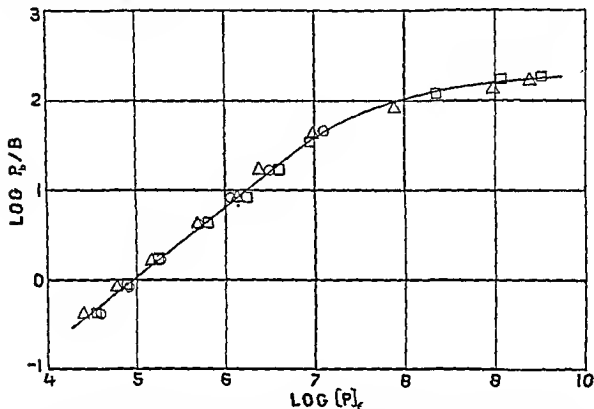


FIG. 4. Phage distribution between heat-killed, susceptible bacteria and broth. Three different  $B$  preparations. Data plotted as adsorption isotherm. Contact period 16 hours at 10°C.

- Δ  $B$  preparation No. 1  
 □ " " " 2  
 ○ Data of Table VI
- } Data of Table VII

reversible while no evidence of reversal could be obtained in the present instance. It was finally found that peculiar results of this sort, giving distribution plots midway between those of normal and associative distribution, depended upon some substance present in unwashed cultures. This material, derived from the medium

<sup>1</sup> In order to apply Langmuir's equation quantitatively it is necessary to make arbitrary assumptions as to the constants owing to lack of experimental data, so that no definite significance would attach to the calculations.

upon which the bacteria were grown and amboceptor-like in action, furthered the binding of  $P$  by dead  $B$  and was shown in control experiments with ultrafiltrates not to exert any inactivating action upon phage. Its presence in  $P$ -live  $B$  mixtures used for distribution experiments resulted in the  $B$  taking up considerably more  $P$  than is normally the case and was responsible for several apparently anomalous series until the cause was discovered. In subsequent adsorption and distribution experiments, the  $B$  were washed several times with broth or saline to remove the disturbing factor. When new lots of agar (of the same formula) were used for growing the bacteria no differences were found between washed and unwashed  $B$ , both giving strictly regular results.

It is quite possible occasionally to obtain what appear to be fairly good adsorption isotherms when the ratio  $P/B$  of the entire series is

TABLE VIII

*Irreversibility of Phage Sorption by Heat-Killed, Susceptible Bacteria*  
 Temperature  $10^{\circ}\text{C}$ . Total  $B$   $2.4 \times 10^8$  Volume 8 Ml. Initial Period of Contact  
 16 Hours.  $B$  Killed at  $65^{\circ}\text{C}$ .

|  | Total initial<br>P.U. | $[P]_e$           | Total<br>extracellular<br>P.U. | $K = \frac{(P_b/B)(25 \times 10^{10})}{[P]_e}$ |
|--|-----------------------|-------------------|--------------------------------|--|
| (1) $P$ - $B$ mixture for establishing $[P]_e$ after 16 hours.   | $4 \times 10^9$       | $3 \times 10^6$   | $2.4 \times 10^7$              | $1,380 \times 10^3$                            |
| (2) Reversal experiment<br>At equilibrium (16 hours) diluted to 80 ml. with broth and shaken 24 hours at $10^{\circ}\text{C}$ . Final volume $10 \times$ initial volume. | "                     | $2.1 \times 10^5$ | $1.7 \times 10^7$              | $19,800 \times 10^3$                           |

high and if lower  $P/B$  ratios are not included in the test the results may be misleading. However, the plotted points do not give a smooth curve, the slope is very low and other experiments including lower  $P/B$  ranges indicate that the region is one of  $B$  saturation with  $P$ .

Apparently, then, the combination of  $P$  with dead  $B$  may be expressed in the form of the usual adsorption isotherm equation except in cases of very high  $P/B$  ratios. If the process is a true adsorption and proceeds to  $> 99$  per cent completion as in the present instance, it should be very difficult to demonstrate any reversibility. This

point was tested experimentally by running at the same time as the reversal experiment with live *B*, a similar series employing dead *B*. Table VIII gives the results obtained and clearly demonstrates that no detectable reversal has taken place. Other experiments performed at 10°C. and 0°C. with various  $[P]$ 's and  $[B]$ 's lead to the same general conclusion.

The control tubes for total  $[P]$  in distribution experiments performed with dead *B* invariably give a  $[P]$  value identical with or less than that of the supernatant fluid left after centrifugation of *B*, although these determinations are made with dilutions of the entire *P-B* mixture. This apparent paradox is readily explicable on the basis of the foregoing facts concerning *P* adsorption. *P* taken up by dead cells is retained by them and consequently does not function in the *P* titration. Hence the  $[P]$  of the *P-B* mixture ordinarily will be that of the supernatant. However, if low dilutions of the *P-B* suspension are employed in the titration set-up an appreciable concentration of dead *B* may be introduced. Now, as the live organisms of the test series grow and produce *P* the latter will be adsorbed by the dead *B* in considerable amounts, thereby increasing time of lysis and causing the mixture to have an apparent  $[P]$  less than the free  $[P]$  at equilibrium.

The fact that the combination of *P* with dead *B* is non-reversible brings up the possibility that the phenomenon studied may be not one of adsorption but rather an inactivation of *P* by some hypothetical substance derived from the dead *B* and set free in the broth. This possibility was tested by filtering heavy suspensions of dead *B* in broth and saline through acetic-collodion membranes (14) just dense enough to retain all organisms while allowing free passage of substances in solution and adding the filtrates to known  $[P]$ 's. The filtrate-*P* mixtures were shaken at 10°C. and 35°C. for 16 hours and the  $[P]$  of the liquid then determined. In no instance was it possible to detect the least loss in *P* titre, showing that dead *B* remove *P* from solution by a process of *P-B* combination and not by liberating any soluble inactivating substance.

*The Effect of Varying Temperatures at Which B Are Killed on B Adsorptive Capacity.*—This relationship was investigated by exposing aliquots of the same *B* preparations for 3 hours to temperatures of 55°C., 85°C. and 105°C. The heat-killed bacteria were then tested in

upon which the bacteria were grown and amboceptor-like in action, furthered the binding of  $P$  by dead  $B$  and was shown in control experiments with ultrafiltrates not to exert any inactivating action upon phage. Its presence in  $P$ -live  $B$  mixtures used for distribution experiments resulted in the  $B$  taking up considerably more  $P$  than is normally the case and was responsible for several apparently anomalous series until the cause was discovered. In subsequent adsorption and distribution experiments, the  $B$  were washed several times with broth or saline to remove the disturbing factor. When new lots of agar (of the same formula) were used for growing the bacteria no differences were found between washed and unwashed  $B$ , both giving strictly regular results.

It is quite possible occasionally to obtain what appear to be fairly good adsorption isotherms when the ratio  $P/B$  of the entire series is

TABLE VIII

*Irreversibility of Phage Sorption by Heat-Killed, Susceptible Bacteria*  
 Temperature  $10^{\circ}\text{C}$ . Total  $B$   $2.4 \times 10^8$  Volume 8 *Ml*. Initial Period of Contact  
 16 Hours.  $B$  Killed at  $65^{\circ}\text{C}$ .

|  | Total initial<br>P.U. | $[P]_e$           | Total<br>extracellular<br>P.U. | $K = \frac{(P_b/B)(25 \times 10^{10})}{[P]_e}$ |
|--|-----------------------|-------------------|--------------------------------|--|
| (1) $P$ - $B$ mixture for establishing $[P]_e$ after 16 hours.   | $4 \times 10^9$       | $3 \times 10^5$   | $2.4 \times 10^7$              | $1,380 \times 10^3$                            |
| (2) Reversal experiment<br>At equilibrium (16 hours) diluted to 80 <i>ml</i> . with broth and shaken 24 hours at $10^{\circ}\text{C}$ . Final volume $10 \times$ initial volume. | "                     | $2.1 \times 10^5$ | $1.7 \times 10^7$              | $19,800 \times 10^3$                           |

high and if lower  $P/B$  ratios are not included in the test the results may be misleading. However, the plotted points do not give a smooth curve, the slope is very low and other experiments including lower  $P/B$  ranges indicate that the region is one of  $B$  saturation with  $P$ .

Apparently, then, the combination of  $P$  with dead  $B$  may be expressed in the form of the usual adsorption isotherm equation except in cases of very high  $P/B$  ratios. If the process is a true adsorption and proceeds to  $> 99$  per cent completion as in the present instance, it should be very difficult to demonstrate any reversibility. This

TABLE IX

Summary of Reaction Velocity Experiments on: (a) Combination of Live, Resting, Susceptible Bacteria with Phage  
(b) Combination of Heat-Killed, Susceptible Bacteria with Phage

$[P]_0 = 1 \times 10^3$  P.U./Ml. throughout.  $[B]_0 = 1 \times 10^8$  B./Ml. throughout. Temperature  $10^\circ\text{C}$ .

$K$ 's Are Those of Calculated Curves Best Fitting Experimental Data

| <i>B</i> preparation used: Live, resting, susceptible <i>B</i> |  | <i>B</i> preparation used: Heat-killed, susceptible <i>B</i> |  |
|--|--|--|--|
| Medium   | $K$ calculated from<br>$k = \frac{1}{t[B]} \ln \frac{([P]_0 - [P]_t)}{([P]_0 - [P]_t)}$                          | Medium   | $K$ calculated from<br>$k = \frac{1}{t[B]} \ln \frac{([P]_0 - [P]_t)}{([P]_0 - [P]_t)}$                          |
| Broth  | $\left. \begin{array}{l} \text{with shaking} \\ \text{without shaking} \end{array} \right\} \text{Run together}$ | Broth  | $\left. \begin{array}{l} \text{with shaking} \\ \text{without shaking} \end{array} \right\} \text{Run together}$ |
| "  |  | "  |  |
| "  |  | "  |  |
| Broth  | $\left. \begin{array}{l} \text{with 20 per cent} \\ \text{glycerine} \end{array} \right\} \text{Run together}$   | Broth  | $\left. \begin{array}{l} \text{with 20 per cent} \\ \text{glycerine} \end{array} \right\} \text{Run together}$   |
| "  |  | "  |  |
| "  |  | "  |  |
| Average  | .0142  | Average  | .0143  |

The close agreement of the average  $K$ 's is obviously fortuitous.



the usual way for ability to adsorb phage. No significant changes were found over this range of temperatures.

## II. REACTION VELOCITY

In order to obtain some idea of the course of events between the time of mixing phage with susceptible bacteria and the final attainment of equilibrium the reaction velocity of the *P-B* system was investigated, using both live and dead bacteria.

*Methods:* The live bacterial reactant was prepared as outlined under Distribution of phage between living susceptible bacteria and broth in the absence of growth. Appropriate concentrations of *P* and *B*, previously cooled to 10°C., were mixed and the mixtures were shaken at 10°C. Samples taken at intervals were centrifuged at high speed 0.1 hour in iced cups. The supernatants were pipetted off, diluted with 0°C. broth and kept at 0°C. until they could be titrated.

In the case of the reaction of *P* with dead *B* the latter reactant was made up according to the procedure described in Distribution of phage between heat-killed susceptible bacteria and broth. Sampling and titrating were carried out as above.

The removal of *P* from solution by resting live bacteria or by heat-killed cells proceeds very rapidly and the time of sampling must be accurately fixed to obtain reliable data. Consequently the middle of the centrifuging period was arbitrarily designated as *t*-sampling in these experiments. The initial [*B*] used throughout was  $1 \times 10^8$  *B*/ml. while initial [*P*] was  $1 \times 10^8$  P.U./ml.

The distribution of *P* between live, susceptible *B* and broth in accordance with the Law of Normal Distribution would suggest that the combination of *P* with *B* involves diffusion of the lytic substance into the cell. It is not surprising therefore to find that the rate of the process, in common with those found for many other cases of diffusion into living cells, is proportional to the concentration gradient and to the number of cells present.

That is:

$$dP/dt \propto ([B]) ([P]_t - [P]_e),$$

where [*B*] = concentration of bacteria, [*P*]<sub>*t*</sub> = free *P*/ml. at any time *t* and [*P*]<sub>*e*</sub> = concentration of *P* at equilibrium.

It follows that

$$dP/dt = k ([B]) ([P]_t - [P]_e),$$

which on integration gives:

$$k = \frac{1}{t [B]} \ln \frac{([P]_0 - [P]_e)}{([P]_t - [P]_e)}.$$

TABLE IX  
 Summary of Reaction Velocity Experiments on: (a) Combination of Live, Resting, Susceptible Bacteria with Phage  
 (b) Combination of Heat-Killed, Susceptible Bacteria with Phage  
 $[P]_0 = 1 \times 10^8$  p.v./ml. throughout.  $[B]_0 = 1 \times 10^8$  B/ml. throughout. Temperature  $10^\circ\text{C}$ .  
 $K$ 's Are Those of Calculated Curves Best Fitting Experimental Data

| B preparation used: Live, resting, susceptible B |   | B preparation used: Heat-killed, susceptible B |   |
|--|---|--|---|
| Medium   | $K$ calculated from<br>$k = \frac{1}{t[B]} \ln \frac{([P]_0 - [P]_t)}{([P]_i - [P]_t)}$ | Medium   | $K$ calculated from<br>$k = \frac{1}{t[B]} \ln \frac{([P]_0 - [P]_t)}{([P]_i - [P]_t)}$ |
| Broth  | $0.0139 \times 10^{-8}$   | Broth  | $0.0142 \times 10^{-8}$   |
| " with shaking                                   | .0148   | " with shaking                                 | .0158   |
| " without shaking                                | .0156   | " without shaking                              | .0170   |
| Broth  | .0148   | Broth  | .0127   |
| " with 20 per cent<br>glycerine                  | .0138   | " with 20 per cent<br>glycerine                | .0110   |
| Broth  | .0125   | Broth  | .0150   |
| Average  | .0142   | Average  | .0143   |

The close agreement of the average  $K$ 's is obviously fortuitous.

Fig. 5 shows graphically a single experiment performed in duplicate with live and dead *B*. There is no significant difference in the reaction velocities. This in general expresses the results of the series of velocity experiments (cf. Table IX), *i.e.*, live, resting, susceptible *B* and heat-killed, susceptible *B* take up *P* from solution at much the same rate, although it should be noted that equilibrium is rapidly reached

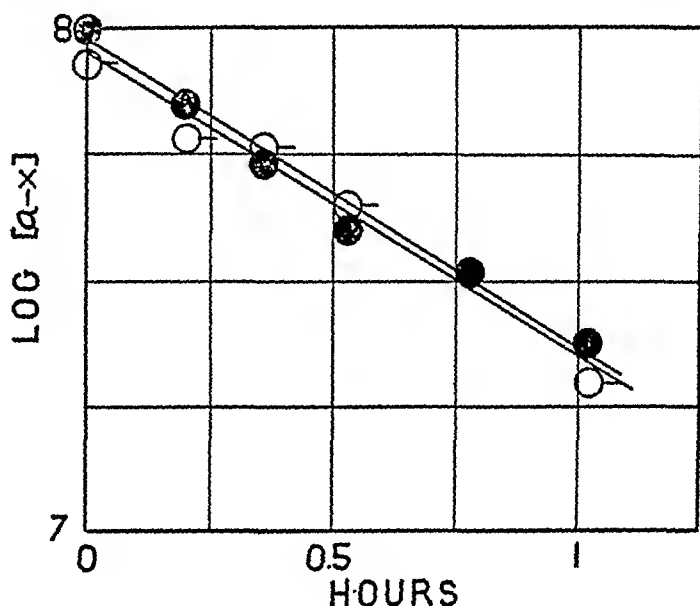


FIG. 5. Velocity of reactions { a. Live, resting, susceptible *B* + *P*  
 { b. Heat-killed, susceptible *B* + *P*  
 $[B]_0 = 1 \times 10^8$      $[P]_0 = 1 \times 10^8$     Temperature  $10^\circ\text{C}$ .     $(a - x) = [P]_{\text{free}}$   
 after time  $t$ .

○ Live *B* curve (lower line)

● Dead *B* " (upper " )

No significant difference is seen in the velocities of the two reactions.

with live cells whereas dead bacteria continue reacting with *P* until much more of the latter is removed from solution.

In order to determine whether the period consumed in the reaction represented delay due to diffusion of *P* to the *B* cell or time utilized in a reaction between *P* and *B*, once contact was established, the following experiments were performed:

1. Duplicate set-ups with *P* and dead *B* were made. One series was shaken vigorously at  $10^\circ\text{C}$ . while the other was not shaken.

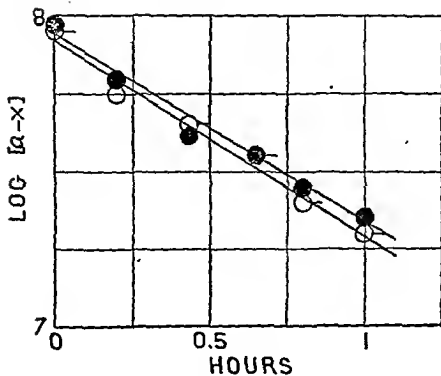


Fig. 6a

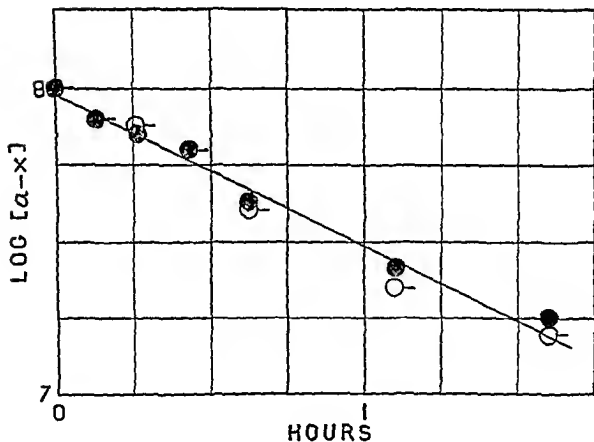


Fig. 6b

FIG. 6. Influence of doubling viscosity upon velocity of reactions:

a. Live, resting, susceptible  $B + P$

b. Heat-killed, susceptible  $B + P$

$[B]_0 = 1 \times 10^8$   $[P]_0 = 1 \times 10^5$  Temperature  $10^\circ\text{C}$ .  $(a - x) = [P]_{\text{free}}$  after time  $t$ .

○  $B + P$  normal viscosity of broth.

●  $B + P$  viscosity of medium doubled with twenty per cent glycerine.

There is no marked difference in the velocities of the reactions attributable to the increased viscosity in either set of experiments.

and that  $[P]$  determinations will detect degrees of  $B$  growth otherwise easily overlooked if direct estimation of  $[B]$  is relied upon. The value of such an indicator is obvious when experimental requirements necessitate maintenance of live  $B$  at a constant  $[B]$  level. The sensitivity of  $[P]$  to  $B$  reproduction rests upon a previously described relationship (9) existing between the rates of  $P$  production and  $B$  reproduction; namely, that  $dP/dt$  is proportional to a power of the rate  $dB/dt$ . Therefore very small increments in  $[B]$  give rise to measurable increases in  $[P]$ .

The second point of interest relates to  $[P]$  determinations in the presence of dead  $B$ . Total  $[P]$  determinations of  $P$ -dead  $B$  mixtures invariably give values identical with or less than the  $[P]$ 's of the supernatants left after centrifugation of  $B$ . The obvious conclusion is that  $P$  taken up by dead cells is retained by them in an inactive form and consequently does not function in the  $P$  titration. The experimental demonstration of the irreversibility of  $P$  adsorption by dead  $B$  substantiates this view, and with this in mind one would expect the total  $[P]$  of a  $P$ -dead  $B$  mixture to be that of the supernatant—as is actually true. If, however,  $[B]$  is high or if low dilutions of a  $P$ - $B$  mixture are used in running the titration a considerable number of dead  $B$  may be introduced into the titration set-up. As the test  $B$  grow and produce  $P$  appreciable amounts of the latter will be adsorbed by the dead cells. Since lysis depends upon the attainment of a particular  $P/B$  ratio (9) the time of lysis will be prolonged and the mixture will appear to have a total  $[P]$  less than the free  $[P]$  at equilibrium.

The mixtures of known  $[P]$ 's with known concentrations of live  $B$  used as controls to detect  $B$  growth in distribution experiments with live non-growing  $B$  served also as useful indicators of  $B$  death. Dead  $B$  take up relatively large amounts of  $P$  irreversibly and since such  $P$  does not function in the titration of  $P$ - $B$  mixtures for total  $[P]$  determinations, it is clear that any large number of deaths among the  $B$  population will reduce the apparent  $[P]$  of control tubes. Consequently the control series of  $P$ -live  $B$  mixtures served in this work as satisfactory criteria both of  $B$  growth and  $B$  survival under test conditions.

## SUMMARY AND CONCLUSIONS

The above data relating to the antistaphylococcus phage and single strain of *S. aureus* with which previous papers have been concerned (9, 10, 11, 12), bring out the following points.

(a) For live, resting, susceptible *B* suspended in broth as well as for *B* in a *P-B* mixture during the logarithmic phases of *B* growth and *P* formation, *P* is distributed in a manner typical of numerous materials soluble in both phases of a two phase system, i.e., distribution proceeds in accordance with the equation  $C_b/C_a = K$  where  $C_b$  = extracellular *P*/ml. of broth and  $C_a$  = intracellular *P*/ml. of *B*. The combination is quantitatively reversible.

(b) With heat-killed, susceptible *B*, *P* distribution is of the adsorptive type, expressible in the form of the adsorption isotherm equation  $a = kC^{1/n}$ . The average value of  $1/n$  is 0.80 in agreement with the results of von Angerer (2). Under ordinary conditions dead *B* take up much more *P* than do live *B*, the reaction proceeding to > 99 per cent completion. The combination of *P* with dead *B* is not demonstrably reversible and with high initial *P/B* ratios saturation of *B* with *P* is effected. Bacteria killed at 65°C., 80°C. and 100°C. show no differences in adsorptive ability.

(c) The rates at which live, resting, susceptible *B* and heat-killed, susceptible *B* remove *P* from solution do not differ significantly. Velocity constants of the process calculated from  $k = \frac{1}{t[B]} \ln \frac{([P]_0 - [P]_e)}{([P]_i - [P]_e)}$  agree satisfactorily. It is shown that the time interval consumed is concerned with an actual reaction between *P* and *B* and not with diffusion of *P* through the broth to *B*.

(d) *P* determinations have been found to serve as satisfactory indicators for *B* growth in *P-B* mixtures where  $[B]$  is to be maintained at a constant level. Very small increments in  $[B]$  give rise to measurable increases in *P* by virtue of the fact that  $dP/dt$  is proportional to a power of the rate  $dB/dt$  (9).

(e) Similarly  $[P]$  estimations will detect death of *B* cells in *P*-live *B* suspensions. Dead *B* take up large amounts of *P* irreversibly; such *P* cannot function in the titration and the result is a sharp drop in  $[P]$  of controls.

The writer wishes to thank Dr. John H. Northrop for constant helpful criticism throughout this work.

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## ON THE THEORY OF NERVOUS CONDUCTION

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Although we know as yet very little about the actual mechanism of the propagation of the nervous impulse, it is however generally recognized that this propagation is based on the excitation caused by the action current of the nerve itself.<sup>1</sup> When a nerve is stimulated by an electric current, the excitation occurs in that region, in which the current flows *from* the nerve, and the excited region becomes electronegative with respect to the rest of the nerve. This electronegativity of the region *E* (Fig. 1) generates local bioelectric currents, which at *E* flow *into* the nerve, but in the adjacent regions, *A* and *B*, flow *from* the nerve (Fig. 1). It is assumed that this causes an excitation at *A* and *B* and the excitation will thus spread from *E* in both directions.<sup>2</sup> The tacit assumption is usually made, that this spread of the excitation occurs with constant velocity. However, to our best knowledge, no one has yet established the exact equations which should govern this process and thus justify the assumption. The mathematical treatment of this problem is the purpose of this communication.

In order to establish the desired equations we must possess a mathematical description of the laws of excitation by electric currents. This mathematical description must account for the two fundamental facts, namely:

1. For suddenly established constant currents the relation between the intensity of the current and the time which the current must act in order to cause excitation, is represented by a curve like that shown in Fig. 2;  $i_0$  corresponds to the threshold value of the current, below which no excitation will occur at all.

2. If the current increases from zero sufficiently slowly, it may exceed the threshold value many times without the excitation taking place.



We shall consider two mathematical expressions, both describing these two facts. One is the formula of Hoorweg, which we shall discuss mainly because of its historical importance, and the other is a

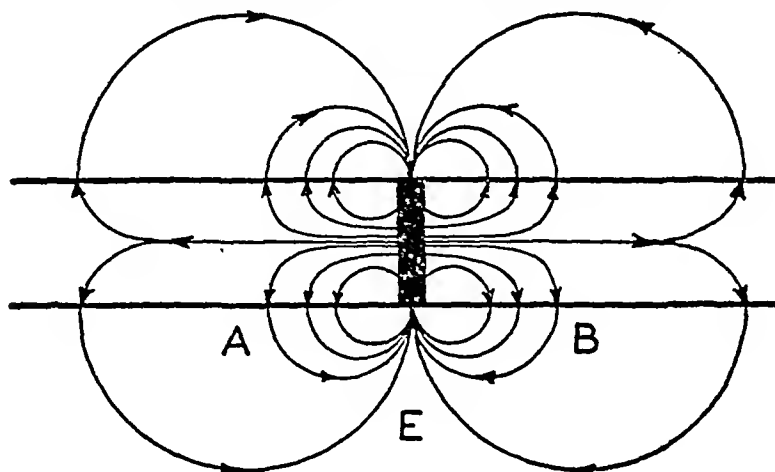


FIG. 1

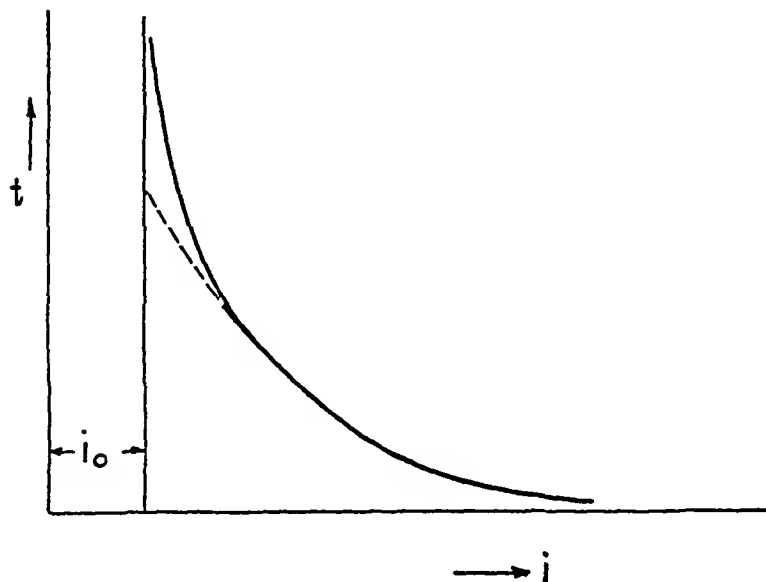


FIG. 2

more recent expression, proposed by L. Lapique.<sup>3</sup> This latter is quite arbitrary, but presents some interesting features.

According to Hoorweg, the exciting current causes the increase of a

certain quantity  $\epsilon$ , whose physical nature is not specified, and which is called "elementary excitation" (Elementarerregung). The actual excitation occurs when  $\epsilon$  reaches a certain value  $\epsilon_0 > 0$ . Hoorweg assumes:

$$\frac{d\epsilon}{dt} = i(t)e^{-\beta t} \quad (1)$$

where  $i(t)$  denotes the current, which is some function of time  $t$ , and  $\beta$  is a constant. Hence:

$$\epsilon(t) = \int_0^t i(t)e^{-\beta t} dt \quad (2)$$

Formula (2) represents, at least quantitatively, the above two facts. For a current which is suddenly established at  $t = 0$  and kept at a constant value  $i$ , we have  $i(t) = i$ , hence:

$$\epsilon(t) = i \int_0^t e^{-\beta t} dt = \frac{i}{\beta} (1 - e^{-\beta t}) \quad (3)$$

Equating the right hand side of (3) to  $\epsilon_0$  we obtain for the  $i - t$  curve the equation:

$$i = \frac{\beta \epsilon_0}{1 - e^{-\beta t}} \quad (4)$$

which is of the desired type, with  $i_0 = \beta \epsilon_0$ .

For a slow linear increase of the current we have  $i = \lambda t$ , which gives:

$$\epsilon(t) = \lambda \int_0^t t e^{-\beta t} dt = \frac{\lambda}{\beta} \left[ \frac{1}{\beta} - \left( t + \frac{1}{\beta} \right) e^{-\beta t} \right] \quad (5)$$

With increasing  $t$ ,  $\epsilon(t)$  tends asymptotically to the value  $\frac{\lambda}{\beta^2}$  and never exceeds this value. If therefore  $\lambda < \beta^2 \epsilon_0$  then  $\epsilon(t)$  will never reach  $\epsilon_0$ .

Now we shall establish the equations for the velocity of propagation of the nervous impulse assuming that Hoorweg's law holds for the excitation. The distribution of the currents around an excited region  $E$  (Fig. 1) will not be uniform. The current flowing from the nerve at

any point will be the weaker, the farther the considered point is away from  $E$ . As to the actual distribution of the current, it will depend on the constitution of the nerve fiber and on the electrical conductivities of various parts of it. Some more or less simplifying hypothesis is therefore unavoidable. If we make the usual assumption, that the nerve consists of a comparatively better conducting core and of a worse conducting sheath, it can easily be shown that the distribution of the current around an excited region  $E$  will be exponential. That is, if  $s$  denotes the distance of a point along the nerve from  $E$  the current caused at this point by the electronegativity of  $E$  and flowing from the nerve, will be given by:

$$i(s) = Ie^{-\alpha s} \quad (6)$$

where  $\alpha$  is a constant depending on the conductivities of the sheath and the core and  $I$  is the current in the immediate neighborhood of  $E$ , which also is a constant for a given nerve. If the "internal" resistance of the excited region  $E$  is small as compared with the resistance of the rest of the nerve,  $\alpha$  and  $I$  will also be independent of the size of the region  $E$  and  $s$  must be counted from its "edge"  $e$  (Fig. 3).

Let the excitation at  $E$ , caused by an external exciting current, have occurred at the time  $t = 0$ . That is, the current distribution described by (6) is established at that time. In order that this bioelectric current should cause an excitation in the immediate surroundings of  $E$ ,  $I$  must exceed the value of  $i_o = \beta\epsilon_o$ . And even then it requires a finite time until the excitation in the neighborhood of  $E$  will occur. This time is found from equation (4) by putting  $i = I$ . It is equal to

$$t_1 = -\frac{1}{\beta} \log \left( 1 - \frac{\beta\epsilon_o}{I} \right) \quad (7)$$

( $t_1$  is positive because the log is negative).

Hence only after this time has past from the moment of establishment of the excitation in  $E$  will this excitation begin to spread in both directions from  $E$ . Since the whole phenomenon is symmetrical with respect to the original position of  $E$ , we shall consider only the propagation in one direction. We shall take as origin of coordinates the point  $E$  at which the nerve is originally stimulated.

Consider any other point along the nerve,  $x_0$ . At the moment  $t = 0$  a current

$$i(x_0) = Ie^{-\alpha x_0}$$

is established at that point (Fig. 3). But as soon as the excitation begins to spread from  $E$ , the current at  $x_0$  begins to increase, because the distance  $s$  from this point to the excited region decreases.

Let the velocity of propagation of the nervous impulse, in other words the velocity of this spread of excitation, be in general some function of time, which we shall denote by  $v(t)$ . It is the shape of this function  $v(t)$  that we wish to determine.

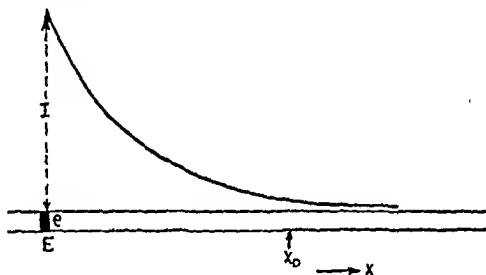


FIG. 3

Since the propagation begins only at the moment  $t = t_1$ , the distance  $s$  of the point  $x_0$  from the edge of the excited region at any time  $t > t_1$  is given by:

$$s = x_0 - \int_{t_1}^t v(t) dt = x_0 - u(t). \quad (8)$$

Hence between  $t = 0$  and  $t = t_1$  the current at  $x_0$  is constant and has the value

$$Ie^{-\alpha x_0}, \quad (9)$$

whereas after  $t = t_1$  the current at  $x_0$  varies with time according to the expression:

$$Ie^{-\alpha x_0 + \alpha u(t)},$$

Hence the elementary excitation is given, according to (2) by:

$$\epsilon(t) = Ie^{-\alpha x_0} \int_0^{t_1} e^{-\beta t} dt + Ie^{-\alpha x_0} \int_{t_1}^t e^{\alpha u(t) - \beta t} dt, \quad (11)$$

or otherwise:

$$\epsilon(t) = Ie^{-\alpha x_0} \left( K + \int_{t_1}^t e^{\alpha u(t) - \beta t} dt \right), \quad (12)$$

where  $K$  denotes the constant quantity

$$\int_0^{t_1} e^{-\beta t} dt = \frac{1}{\beta} (1 - e^{-\beta t_1}). \quad (13)$$

The excitation at  $x_0$  happens at that moment when at this point  $\epsilon(t)$  reaches the value  $\epsilon_0$ . Hence the equation:

$$Ie^{-\alpha x_0} \left( K + \int_{t_1}^t e^{\alpha u(t) - \beta t} dt \right) = \epsilon_0, \quad (14)$$

which can also be written:

$$\frac{\epsilon_0}{I} e^{\alpha x_0} = K + \int_{t_1}^t e^{\alpha u(t) - \beta t} dt, \quad (15)$$

will give us, when resolved with respect to  $t$ , the time at which the excitation will reach the point  $x_0$ . Since we have taken the point  $x_0$  along the nerve quite arbitrary, this means that equation (15) gives for each point the corresponding time, at which the excitation reaches that point. But this in its turn means, that if resolved with respect to  $x_0$ , equation (15) will give us the distance travelled during the time  $t$  by the excitation from the point  $x = 0$ , at which it started. But this distance is equal to:

$$\int_{t_1}^t v(t) dt = u(t). \quad (16)$$

Hence the unknown function  $u(t)$  must be of such a form, that when the equation (15) is resolved with respect to  $x_0$ , the expression of  $x_0$  as a function of  $t$ , thus found, must be again of the form  $u(t)$ . In order

to determine  $u(t)$  we must therefore substitute in (15)  $u(t)$  for  $x_0$  and thus obtain a functional equation:

$$\frac{\epsilon_0}{I} e^{\alpha u(t)} = K + \int_{t_1}^t e^{\alpha u(t) - \beta t} dt. \quad (17)$$

Differentiating this we have:

$$\frac{\epsilon_0 \alpha}{I} e^{\alpha u(t)} \frac{du}{dt} = e^{\alpha u(t) - \beta t} \quad (18)$$

or:

$$\frac{du}{dt} = \frac{I}{\epsilon_0 \alpha} e^{-\beta t} \quad (18)$$

with the initial condition:

$$u(t_1) = 0. \quad (19)$$

Hence:

$$u(t) = \frac{I}{\alpha \beta \epsilon_0} (e^{-\beta t_1} - e^{-\beta t}) \quad (20)$$

and, according to (16):

$$v(t) = \frac{I}{\alpha \epsilon_0} e^{-\beta t}. \quad (21)$$

We thus see that the velocity of propagation gradually drops to zero and that the distance travelled by the excitation approaches asymptotically the value

$$\frac{I}{\alpha \beta \epsilon_0} e^{-\beta t_1} \quad (22)$$

without ever reaching this point. The physical reason for this is obvious: The farther a point is situated from the point  $x = 0$  of the original excitation the slower will the action current at this point increase up to the necessary threshold value, because the more time will be required for the excited region to come close enough to the point in question. Therefore, according to the second fundamental fact mentioned on page 517, the excitation at such a point will never occur.

L. Lapique has however criticised Hoorweg's formula (2) and has proposed another one.<sup>4</sup> Lapique imagined a hydraulic model which represents some phenomena of excitation better than Hoorweg's formula. This model is governed by a system of two differential equations. We shall investigate now, to what conclusions about the velocity of propagation we will be led by applying Lapique's equations to our problem in a purely formal way. The question as to whether these equations can be interpreted physically will be left open. But the remarkable properties of Lapique's model make the following investigation desirable.

We now must consider two quantities,  $h$  and  $h'$ , whose variations are governed by the following equations:

$$\frac{dh}{dt} = -k(h - h') + i(t) \quad (23)$$

$$\frac{dh'}{dt} = k(h - h') \quad (24)$$

where  $k$  denotes a constant and  $i(t)$  is again the exciting current. Excitation occurs when the following equation becomes satisfied:

$$a(p + h) = b(p + h') \quad (25)$$

where  $a$ ,  $b$ , and  $p$  are other constants.

Lapique shows that for  $i(t) = i = \text{constant}$ , and with the initial condition  $h = h' = 0$  for  $t = 0$ , equations (23) and (24) also lead to a curve of a general type represented in Fig. 2. The only difference is that now the curve actually touches the line  $i = i_0$ , without however intersecting it, as shown on Fig. 2 by the dotted line. He shows also that for  $i = \lambda t$  no excitation occurs at all if  $\lambda$  is sufficiently small.

We shall now apply equations (23), (24), and (25) to our problem. From (23) and (24) it follows:

$$\frac{d(h + h')}{dt} = i(t) \quad (26)$$

hence:

$$h' = \int_0^t i(t) dt - h \quad (27)$$

and:

$$\frac{dh}{dt} + k \left( 2h - \int_0^t i(t) dt \right) - i(t) = 0. \quad (28)$$

Similarly to the previous case we have here also a constant retardation time  $t_1$ . Due to the transcendency of the equations involved in the integrals of (23) and (24),  $t_1$  cannot however be expressed in a simple form like (7) in terms of the constants  $k$ ,  $a$ ,  $b$  and  $p$ . But equations (9) and (10) remain valid also in this case, so that we have:

$$\begin{aligned} \int_0^t i(t) dt &= I e^{-\alpha x_0} \int_0^{t_1} dt + I e^{-\alpha x_0} \int_{t_1}^t e^{\alpha u(t)} dt = \\ &= I t_1 e^{-\alpha x_0} + I e^{-\alpha x_0} \int_{t_1}^t e^{\alpha u(t)} dt. \end{aligned} \quad (29)$$

This, introduced in (28), gives:

$$\frac{dh}{dt} + 2kh - \left[ I k e^{-\alpha x_0} \left( t_1 + \int_{t_1}^t e^{\alpha u(t)} dt \right) + i(t) \right] = 0. \quad (30)$$

Hence, if  $C$  denotes a constant of integration<sup>4</sup>:

$$h = C e^{-2kt} + e^{-2kt} \int_0^t \left[ I k e^{-\alpha x_0} \left( t_1 + \int_{t_1}^t e^{\alpha u(t)} dt \right) + i(t) \right] e^{2kt} dt. \quad (31)$$

The function  $i(t)$  has the form (9) for  $t < t_1$  and the form (10) for  $t > t_1$ . Hence the expression in the square brackets has also different forms for  $t < t_1$  and  $t > t_1$ . If we denote these correspondingly by  $[ ]_1$  and  $[ ]_2$  we may write: ( $[ ]$  denoting the expression in the square brackets in general)

$$\int_0^t [ ] dt = \int_0^{t_1} [ ]_1 dt + \int_{t_1}^t [ ]_2 dt = K_1 + \int_{t_1}^t [ ]_2 dt = K_2 + \int_0^t [ ]_2 dt \quad (32)$$

with

$$K_1 = \int_0^{t_1} [ ]_1 dt \text{ and } K_2 = K_1 - \int_0^{t_1} [ ]_2 dt.$$

Since we are interested only in what happens at times  $t > t_1$ , we may take zero as lower limit in the integral in the round brackets in (31),



since this will result only in the addition of a constant to the final result and will not affect the shape of the function  $u(t)$  which we seek. Hence:

$$h = Ce^{-2kt} + e^{-2kt}Ie^{-\alpha x_0} \int_0^t \left[ k \left( t_2 + \int_0^t e^{\alpha u(t)} dt \right) + e^{\alpha u(t)} \right] e^{2kt} dt \quad (33)$$

with

$$t_2 = t_1 - \int_0^{t_1} e^{\alpha u(t)} dt. \quad (34)$$

Transforming in a similar manner the integral in (27) we obtain for  $h'$ :

$$\begin{aligned} h' &= Ie^{-\alpha x_0} \int_0^t e^{\alpha u(t)} dt - Ce^{-2kt} - e^{-2kt}Ie^{-\alpha x_0} \times \\ &\times \int_0^t \left[ k \left( t_2 + \int_0^t e^{\alpha u(t)} dt \right) + e^{\alpha u(t)} \right] e^{2kt} dt + K_3 \end{aligned} \quad (35)$$

where  $K_3$  denotes another constant.

If we now introduce (31) and (35) into (25) we obtain after some rearrangement:

$$e^{\alpha x_0} + P \int_0^t \left[ k \left( t_2 + \int_0^t e^{\alpha u(t)} dt \right) + e^{\alpha u(t)} \right] e^{2kt} dt - Qe^{2kt} \int_0^t e^{\alpha u(t)} dt = K_4 e^{2kt} \quad (36)$$

with

$$P = \frac{1}{C}; \quad Q = \frac{Ib}{(a+b)C} \quad (37)$$

and  $K_4$  another constant, which is algebraically expressed in terms of  $t_2$  and  $K_3$ .

Equation (36), resolved with respect to  $x_0$ , gives now the distance travelled by the excitation as a function of time. Hence for the determination of  $u(t)$  we must again put in (36)  $u(t)$  for  $x_0$  and thus obtain:

$$\begin{aligned} e^{\alpha u(t)} + P \int_0^t \left[ k \left( t_2 + \int_0^t e^{\alpha u(t)} dt \right) + e^{\alpha u(t)} \right] e^{2kt} dt - \\ Qe^{2kt} \int_0^t e^{\alpha u(t)} dt = K_4 e^{2kt}. \end{aligned} \quad (38)$$

Introducing now a new variable:

$$y(t) = e^{\alpha u(t)} \quad (39)$$

and differentiating (38) we obtain:

$$\frac{dy}{dt} + P \left[ k \left( t_1 + \int_0^t y dt \right) + y \right] e^{2kt} - Q \left( y + 2k \int_0^t y dt \right) e^{2kt} = R e^{2kt} \quad (40)$$

with

$$R = 2kK_1. \quad (41)$$

If we now put:

$$\int_0^t y dt = z, \quad y = \frac{dz}{dt}, \quad (42)$$

we obtain after some calculations:

$$\frac{d^2 z}{dt^2} + (P - Q) e^{2kt} \frac{dz}{dt} + k(P - 2Q) e^{2kt} z = (R - Pk t_1) e^{2kt}. \quad (43)$$

When  $z$  is determined from (43) then we obtain also  $u(t)$  from (42) and (39), and all the arbitrary constants involved will be determined by the requirement that for  $t = 0$  at all points of the nerve we must have  $h = h_1 = 0$ .

Although the general integral of (43) is not known, we see that for very large  $t$  (43) reduces to

$$\frac{dz}{dt} + Az = B \quad (44)$$

with

$$A = k \frac{P - 2Q}{P - Q}; \quad B = \frac{R - Pk t_1}{P - Q}. \quad (45)$$

Since Lapique assumes that always  $b > a$ , we see that  $A < 0$ . Hence the solution of (44) for very large  $t$  has the form:

$$z \approx \text{Const.} e^{c^2 t}, \quad \text{with } c^2 = A. \quad (46)$$

This gives for  $u(t)$ :

$$u(t) = \text{Const.} + c^2 t, \quad (47)$$

and for the velocity of propagation  $v(l)$ :

$$v = c^2.$$

We thus see that in this case the velocity tends asymptotically to the constant value  $c^2$ . How rapidly, or in other words, how far from the point of initial stimulation this asymptotic value will be sufficiently closely approached depends upon the values of the constants in Lapique's equations.

#### SUMMARY

Assuming that the propagation of the nervous impulse consists in the excitation of adjacent regions of the nerve by the action current of the already excited region, exact equations for the velocity of such a propagation are established and integrated. The result depends on the assumptions which we make about the laws of excitation. If Hoorweg's law is accepted, it is found that the velocity of propagation decreases exponentially with time, and that there is a limiting distance which the impulse will travel and which cannot be exceeded. If however a set of equations proposed by L. Lapique is assumed to govern the process of excitation, we find that the velocity of propagation asymptotically reaches a constant value.

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# THE ANALYSIS OF BIOLUMINESCENCES OF SHORT DURATION, RECORDED WITH PHOTOELECTRIC CELL AND STRING GALVANOMETER

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## INTRODUCTION

The recent development of photoelectric cells and amplification circuits has made it possible to obtain very accurate intensity measurements of luminescences so weak that eye-comparison methods are quite unreliable. The string galvanometer, with its very short period, permits an accurate record to be obtained even if the luminescence is a flash of very short duration. By photographing the shadow of the string in the usual way, the flashing of fireflies in varying oxygen tensions has been recorded and results will be published in a later paper. Records of rapid changes in light intensity of a suspension of luminous bacteria have also been obtained.

In a preliminary communication (1930) we have described some of the results of our study of flashes of luminescence occurring when the luminous materials, luciferin and luciferase, of an ostracod crustacean, *Cypridina hilgendorfi*, are mixed together.<sup>1</sup> The present paper continues this study. It should be recalled that light production by luminous animals is connected with the oxidation by oxygen in water solution of a substance, luciferin ( $\text{LH}_2$ ), in the presence of another body, luciferase (A). The luciferin is thermostable, diffuses slowly, and oxidizes to a compound, oxyluciferin (L), which can be again reduced by hydrogenation to luciferin. The luciferase, destroyed by heat and colloidal in solution, plays two rôles: one, that of a catalyst, accelerating the oxidation of luciferin; and second, that of supplying molecules which can be excited to luminesce by picking up

<sup>1</sup>The *Cypridina* material was obtained through a grant from the Carnegie Institution of Washington, whose support we gratefully acknowledge.

1, it consists of a photoelectric cell;<sup>2</sup> (the large gas-filled type used in television), an amplifier consisting of two 171A power tubes connected in push-pull, and a high-sensitivity string galvanometer. The values of the resistances and battery voltages are given in the figure. One of the variable 11,000 ohm resistances has both a coarse and a fine adjustment, to permit compensation for inequalities between the two vacuum tubes. The photoelectric cell with its guard ring,  $R$ , is mounted in a wooden box, the interior of which is painted black. There are two openings in the box, one in the top into which a test tube can be fitted, and one in the side. Either opening can be tightly closed when desired. The outside of the box is covered with tin. The amplifier is also enclosed in a tin-covered box, and the leads from the photoelectric cell to the amplifier pass through a tube of copper

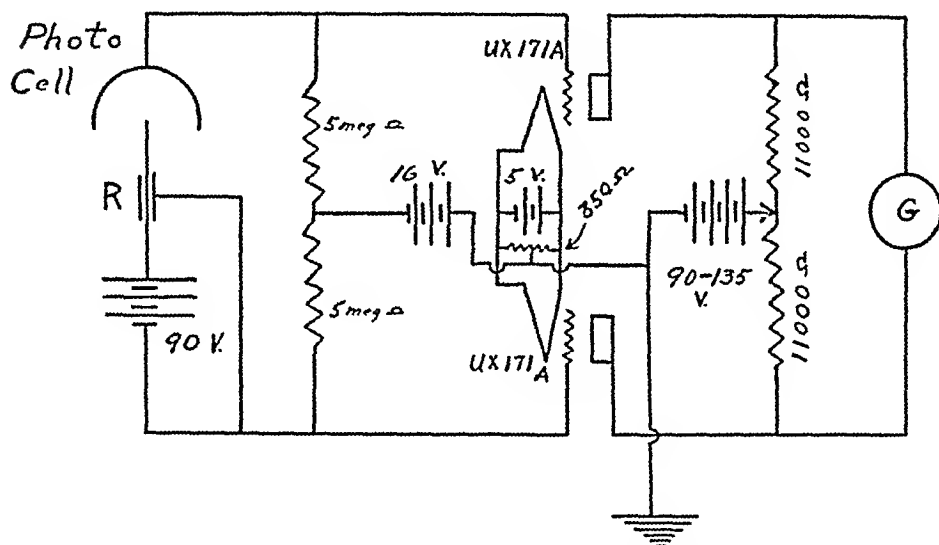


FIG. 1. Amplification system for photoelectric cell. Explanation in text

screening. The leads from the amplifier to the string galvanometer pass through lead tubing, and the entire galvanometer is surrounded by a shield of copper screening. The parts of the shield of the photoelectric cell, amplifier box, galvanometer, and leads are all connected together, but are not grounded. This elaborate shielding was found necessary because of alternating current lines in the vicinity and the extreme sensitivity of the amplifier-galvanometer combination to the pick-up of such disturbances.

<sup>2</sup> We take great pleasure in acknowledging the assistance of the General Electric Company, through Dr. Whitney, and especially of Doctors Hulett, Marvin, and Koller who gave invaluable advice in connection with the photoelectric amplification. The photo cell was a special type kindly supplied by the General Electric Company.

The apparatus was calibrated as follows: a Macbeth illuminometer was set up so that the ground glass screen of the illuminometer fitted into the hole in the side of the photoelectric cell box. A Wratten filter No. 75 was placed in the illuminometer, as this filter passes light of nearly the same spectrum values as that of *Cypridina* luminescence. As indicated in Fig. 2, it was found that there was strict direct proportionality between intensity of illumination (read directly from the inverse scale of the illuminometer) and excursion of the galvanometer string.

For recording the light intensities from *Cypridina* extracts the following scheme was finally adopted: a narrow test tube of 25 cc. capacity was painted black

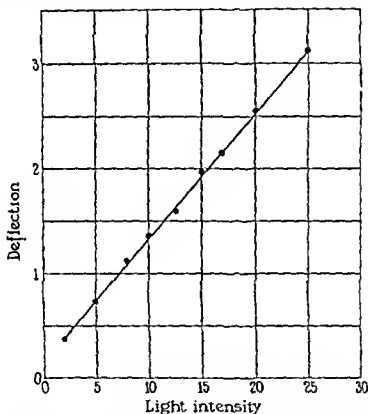
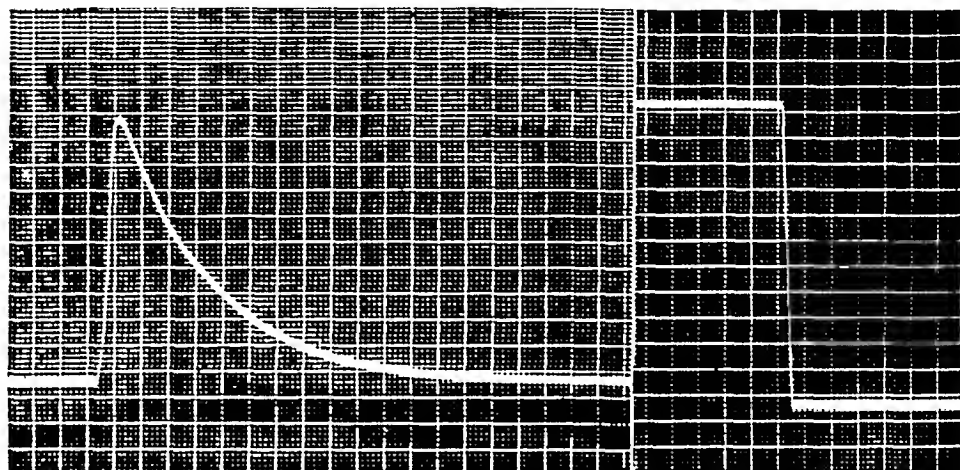


FIG. 2. Linear relation between light intensity and deflection of string of galvanometer. Arbitrary units for ordinates and abscissae.

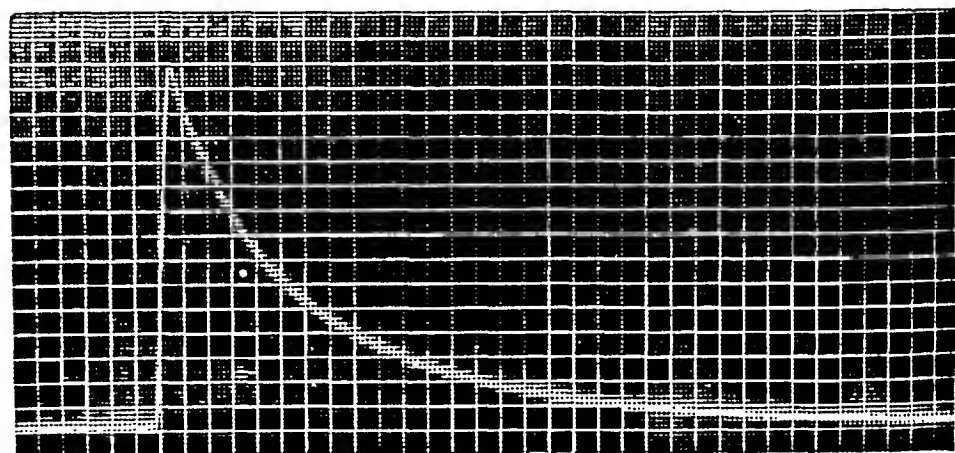
except for a window on the lower third of the tube 15 mm. in length over half the diameter of the tube. About 6 cc. of solution brought the level of the liquid to the top of the window; 10 cc. of luciferin solution were used in every experiment, so that disturbances at the surface due to the force of mixing were prevented from complicating the records. This test tube was firmly mounted in the hole in the top of the photoelectric cell box, so that the window in the tube came opposite the sensitive surface of the cell. A pipette fitted with a rubber bulb was mounted above the test tube in such a way that when the bulb was squeezed the contents of the pipette, luciferase solution, were forcibly ejected into the test tube. This method is quite similar to that used by Amberson for mixing the solutions.

Frequently the initial portion of the record would show jagged irregularities due to uneven mixing. These were largely eliminated by first drawing up luciferase solution into the pipette and then some paraffin oil. The paraffin oil will stay



B

A



C

FIG. 3. String galvanometer records of: *A*, mixing water and ink as described in text; *B* and *C*, luminescences on mixing *Cypridina* luciferin and luciferase. Abscissae give time in 0.2 seconds.

at the bottom of the pipette if its bore is small, and when pushed out first, it serves to put the luciferin solution in the test tube in motion so that very rapid

mixing is going on by the time the luciferase reaches the luciferin and the luminescence suddenly appears. It was found unnecessary to use any stirring device, as the small amounts of solution and the force of ejection from the pipette brought about adequate mixing almost instantaneously.

The time required for complete mixing of two solutions by this method was measured in the following way. A test tube was mounted in a wooden block provided with bores in such a way that a beam of light could be passed through the tube. The block was mounted next to the hole in the side of the photoelectric cell box, and a small lamp set up, the light from which passed through the test tube into the cell. The tube was filled with distilled water and ink was injected into the water, a record being taken of the speed with which the mixing took place as shown by the decrease in light passing through the solution. As shown in the record, Fig. 3 A, mixing is complete in 0.06 seconds. The average of all such experiments was 0.055 seconds. When mixing is complete some light still passed the ink so that the record really pictures the time for homogeneous mixing. The period of the string galvanometer at the voltage and tension used was about 0.007 seconds.

The extracts were always prepared fresh for each day's experiments, and used at 23-24°C. All the luciferase solution needed for the day was prepared at one time; the luciferin extracts were made separately for each experiment, since luciferin oxidizes spontaneously in air. Concentrations used are specified for each experiment in the terms of grams of dry powdered *Cypridina* to 11 cc. of solution. The extracts were prepared in the usual manner: luciferin, by pouring boiling water onto the dried powdered material and immediately cooling rapidly under the tap; luciferase, by grinding the animal material in a mortar with the necessary amount of distilled water, allowing to stand some minutes, then filtering. Luciferase solution also contains oxyluciferin.

## RESULTS

Figs. 3 B and C, give reproductions of records from typical experiments. It will be noted that there is a period during which the light is developing to its maximum brightness. In most cases this period is longer than 0.06 seconds, the time found experimentally to be required for mixing; hence it is possible to begin the analysis of the curve at the point of maximum light intensity, since mixing is complete and the solution is homogeneous before the decay of the light begins. The development of luminescence is too short for analysis.

The ordinates on the photographic records are drawn on at the time of exposure by rulings on the cylindrical lens of the camera. The abscissae are imposed on the records by a revolving wheel timed by an electrically driven tuning fork, the ratio being such that the distance



between each white line represents  $\frac{1}{25}$  of a second; the distance between the heavy white lines represents  $\frac{1}{5}$  of a second. The accuracy of this timing device was tested periodically against 60 cycle alternating current. In the graphs of the records the actual time elapsed is given; arbitrary units of light intensity are used as ordinates, since it is unnecessary to know the absolute values.

The graphical analysis of two typical records is shown in Fig. 4, in which log light intensity is plotted against time. These illustrate the two classes into which the analysis of the decay curves group them-

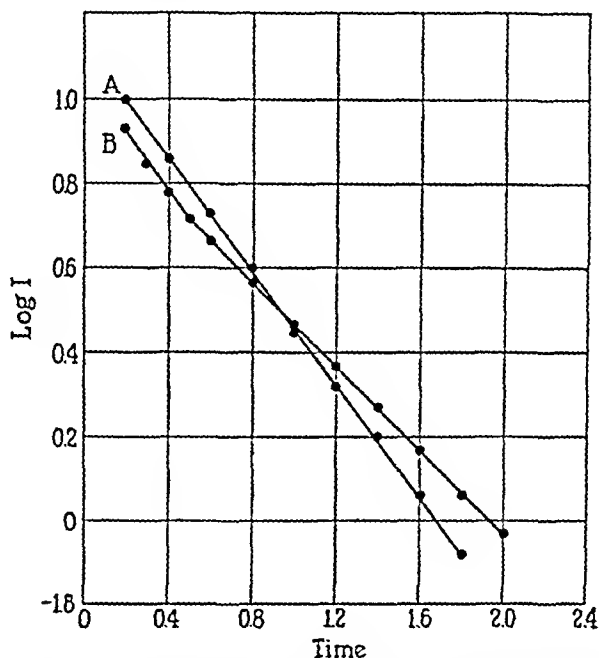


FIG. 4. Log luminescence intensity in arbitrary units plotted against time in seconds for luminescences without (A) and with (B) initial flashes.

selves. A shows an experiment in which there was an initial flash, B shows an experiment in which no initial flash appeared. It was found for one sample of *Cypridina* powder that the initial flash appears whenever the concentration of total luciferin is more than five times that of the luciferase. For another sample the flash appeared when the ratio was less than five. As we have no way of telling what the absolute amount of luciferase or total luciferin is, this may merely mean that the proportions of these substances differed in the second sample,

which was collected in a different locality. It must be recalled that throughout this paper "concentration" of luciferase or luciferin is always expressed as: grams dry *Cypridina* powder used to prepare luciferase (or luciferin) per cc. solution. By total luciferin,  $L_T$ , is meant luciferin ( $LH_2$ ) + oxyluciferin ( $L$ ),—i.e. luciferin both in reduced and in oxidized form. Luciferase (A) solution, because of its method of preparation, always contains some oxyluciferin which must be added in to calculate the total  $L_T$ .

The initial flash is undoubtedly a reality. It lasts much longer than the disturbances connected with mixing the solutions and is the expression of a much greater velocity than corresponds to the subse-

TABLE I  
*Effect of Enzyme (Luciferase) Concentration*

| Experiment number | Luciferin concentration | Luciferase concentration | Total luciferin concentration | Slope | Ratio of enzyme concentration | Ratio of slopes |
|-------------------|-------------------------|--------------------------|-------------------------------|-------|-------------------------------|-----------------|
| F 11              | 0.10                    | 0.01                     | 0.11                          | 10.0  | 0.5                           | 0.48            |
| F 12              | 0.10                    | 0.005                    | 0.105                         | 4.8   |                               |                 |
| E 31              | 0.05                    | 0.04                     | 0.09                          | 56.4  | 0.25                          | 0.298           |
| E 35              | 0.05                    | 0.01                     | 0.06                          | 16.8  |                               |                 |
| E 36              | 0.025                   | 0.01                     | 0.035                         | 29.0  | 0.5                           | 0.414           |
| E 24              | 0.025                   | 0.005                    | 0.03                          | 12.0  |                               |                 |
| E 25              | 0.025                   | 0.0025                   | 0.0275                        | 7.4   | 0.25                          | 0.255           |

quent decay. If not marked, i.e. if of short duration, the log intensity of the flash plotted against time appears to be a straight line, but if the ratio of total luciferin to luciferase is very large (10 to 20) the slope of log initial flash against time is curved and merges into the subsequent reaction whose log intensity against time is also not a straight line, but concave to the time axis. These curves are not such as to indicate a bimolecular process. They are similar to conditions observed in enzyme reaction in general where additional factors, such as percentage of catalyst covered by substrate, come into play, when substrate concentration is high compared to that of enzyme.

Accordingly, we have turned to conditions where ratio of luciferin to luciferase is small and where the plot of log intensity against time

gives a straight line. Under these conditions we can confirm Amberson's and Stevens' findings that the velocity constant (slope of the *log light intensity—time* plot) is very nearly proportional to the concentration of the enzyme, luciferase. It is interesting to find that this relation holds approximately, as indicated in Table I, even when the luminescence is of a few seconds duration.

The anomaly which still appears has to do with the effect of luciferin concentration, which should not affect the velocity constant provided we are dealing with a homogeneous unimolecular reaction in which the light intensity is a measure of the rate of oxidation of luciferin.

TABLE II  
*Effect of Substrate (Luciferin) Concentration*

| Experiment number | Luciferin concentration | Luciferase concentration | Total luciferin concentration | Slope | Slope $\times$ dilution of luciferase |
|-------------------|-------------------------|--------------------------|-------------------------------|-------|---------------------------------------|
| X 2               | 0.025                   | 0.02                     | 0.045                         | 50    | 50                                    |
| E 5               | 0.05                    | 0.02                     | 0.07                          | 29.5  | 29.5                                  |
| E 12              | 0.013                   | 0.02                     | 0.033                         | 53.3  | 53.3                                  |
| E 15              | 0.00625                 | 0.02                     | 0.026                         | 67    | 67                                    |
| E 16              | 0.00625                 | 0.012                    | 0.018                         | 45.5  | 76                                    |
| E 21              | 0.0125                  | 0.01                     | 0.0025                        | 35    | 70                                    |
| E 23              | 0.0125                  | 0.0025                   | 0.015                         | 11    | 88                                    |
| E 33              | 0.0125                  | 0.04                     | 0.0525                        | 90    | 45                                    |

Table II shows the effect of luciferin (substrate) concentration on the velocity constants of the reaction. It is evident from an inspection of the table that the velocity constant is decidedly not independent of the luciferin concentration. Amberson found similarly in some of his experiments that "the more dilute solution appeared, in all but one case, to fall through the decay curve with a faster reaction velocity than its companion concentration solution." He then reports some experiments in which he decreased the concentration of luciferin by accelerating with heat its spontaneous oxidation. In these cases the velocity constant was found to be nearly independent of the luciferin concentration (*i.e.*, independent of the degree of spontaneous oxidation which has taken place). Stevens' tables show a like relationship, the less concentrated substrate showing a larger velocity constant for the same enzyme concentration. In these experiments the concentrations

were varied by using different amounts of whole animal powder in making the extracts.

Amberson's experiments suggest that when total luciferin + oxy-luciferin are constant (as would be the case if the spontaneous oxida-

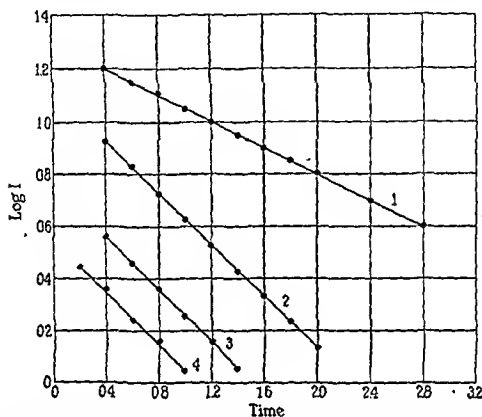


FIG. 5. Log luminescence intensity in arbitrary units plotted against time in seconds for equal luciferase concentrations but varying luciferin and oxy-luciferin concentration as given in Table III.

TABLE III  
*Effect of Total Luciferin Concentration*

| Experiment | Enzyme concentration | Luciferin (initial) | Oxyluciferin (initial) | Total luciferin | Slope |
|------------|----------------------|---------------------|------------------------|-----------------|-------|
| 1          | 0.02                 | 0.05                | 0                      | 0.07            | 25    |
| 2          | 0.02                 | 0.0125              | 0.0125                 | 0.045           | 50    |
| 3          | 0.02                 | 0.025               | 0                      | 0.045           | 49    |
| 4          | 0.02                 | 0.0125              | 0.0125                 | 0.045           | 50    |

tion of luciferin were accelerated by heat), the velocity constant is the same for different concentrations of luciferin, and the records of Fig. 5 prove this point. They were made by mixing luciferin and oxy-

luciferin solutions in the proportions indicated in Table III. Note that whenever the concentration of *luciferin* + *oxyluciferin* is kept constant, the velocity constant (slope) does not change, despite wide variations in the proportions of luciferin and oxyluciferin, which only affect the intercept on the intensity axis. It is total luciferin + oxyluciferin, ( $L_T$ ), which determines the constant,  $k$ , and not luciferin ( $LH_2$ ) concentration.

The question next arises as to how the velocity constant varies as total luciferin + oxyluciferin concentrations vary. This relationship is shown graphically in Fig. 6, a graph based on six series of experiments

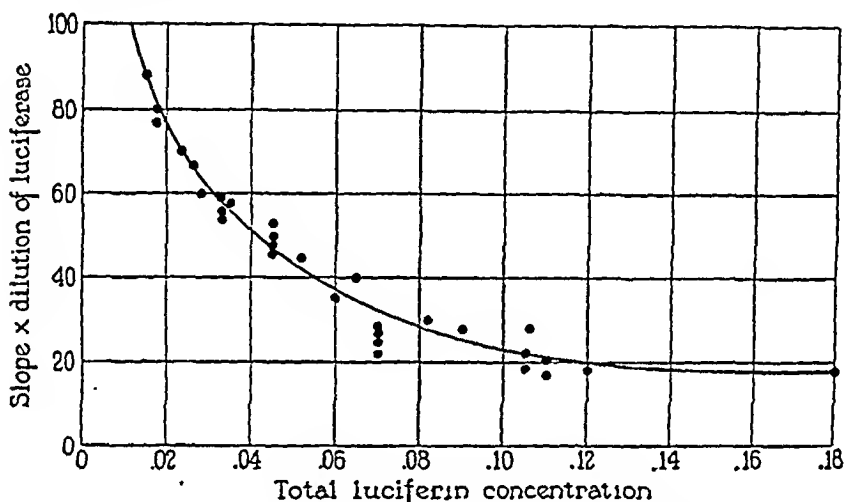


FIG. 6. Relation between velocity constant (slope of log intensity—time plots allowing for luciferase concentration) and total luciferin ( $L_T$ ) (luciferin + oxyluciferin) concentration.

totaling 38 records, covering a range of substrate concentrations from 0.2 gm. per 11 cc. to 0.00625 gm. per 11 cc., and enzyme concentrations ranging from 0.01 to 0.0025 gm. per 11 cc. Since the velocity constant has been shown to be proportional to the enzyme concentration, it seemed advisable in studying the effect of total luciferin concentration to use varying concentrations of enzyme and then to multiply the experimentally determined velocity constants by a factor correcting for the degree of dilution of the enzyme, as in this way the relation could be more thoroughly tested. Slope is plotted against total luciferin + oxyluciferin concentration, and it will be observed that with concen-

trated total luciferin the slope is nearly independent of the total luciferin ( $L_T$ ), whereas with dilute  $L_T$  the slope becomes increasingly steeper.

The curve is of such a nature that the slope is inversely proportional to the  $\sqrt{L_T}$  over all except the range with high ratio of  $\sqrt{L_T}$  to luciferase; that is, slope =  $K \frac{1}{\sqrt{L_T}}$ . One can best see the relationship by plotting the reciprocal of the  $\sqrt{L_T}$  against the velocity constants (slopes). A straight line is obtained with some deviation in the high  $L_T$  concentration region, as shown in Fig. 7.

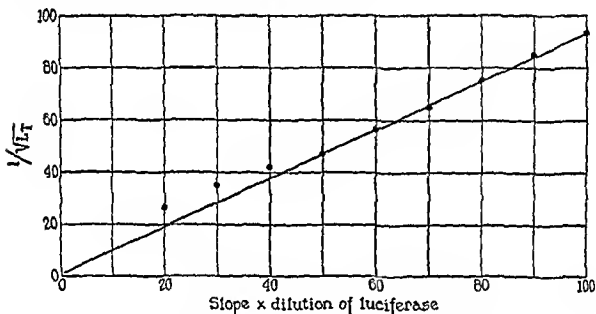


FIG. 7. Velocity constants of Fig. 6 plotted against reciprocal of square root of total luciferin + oxyluciferin ( $L_T$ ) concentration.

The general form of the curve is similar to adsorption curves and immediately suggests that the slope measures the adsorption of luciferin and oxyluciferin on luciferase. In addition, both the luciferin and oxyluciferin must follow the *same* adsorption isotherm. Once adsorbed on luciferase, we have the luminescence intensity proportional to luciferin concentration and falling off logarithmically according to the unimolecular equation. We must further assume that only the adsorbed luciferin molecules are activated and can oxidize, giving rise to luminescence. The velocity constant is a measure of the per cent of change in unit time, so that we may select the unit of time as that when half the adsorbed molecules have reacted. If 100 molecules

are present and  $\sqrt{100} = 10$  are adsorbed, five will react in one unit of time and the oxidation will be  $\frac{5}{100}$ . With half the concentration, 50 molecules,  $\sqrt{50} = 7 +$  will be adsorbed and 3.5 molecules in 50 will react in unit time or  $\frac{7}{100}$ , a higher velocity. All the luciferin will finally react, since we must consider that both luciferin and oxy-luciferin are continually stepping on and off the luciferase surface. At the end of the reaction there are still ten molecules adsorbed from the solution with 100 molecules present and seven molecules adsorbed from the solution with 50 molecules present, but *they are all oxy-luciferin molecules*. The luminescence has ceased. The velocity constant is greater with more dilute  $L_T$  for the same reason that the velocity constant is greater the higher the temperature, because of the greater proportion of activated molecules, activated in this case by a change in the adsorption equilibrium. Here we have a rather unique case of the same adsorption equilibrium of reactant and resultant giving us always the unimolecular curve, but a velocity constant which varies with the concentration of  $L_T$  and in fact measures the adsorption of  $L_T$  on the catalytic surface.

#### DISCUSSION

We are aware that the change of slope with changing total luciferin concentration may have another explanation than that of adsorption, namely, the formation of enzyme-substrate compounds, a suggestion already made for the case of enzymic inversion of cane sugar.

Michaelis and Menten (1913) have explained the deviations of invertase kinetics by assuming that the cane sugar forms a compound with invertase, the rate of reaction depending on the concentration of this enzyme-substrate compound rather than on substrate concentration. For the same concentration of enzyme and varying concentrations of cane sugar, the initial velocity of inversion is determined by an equilibrium as follows:—

Substrate ( $S$ ) + enzyme ( $E - c$ ) = enzyme-substrate ( $c$ ), in which  $E$  = total enzyme and  $c$  = combined enzyme. From the law of mass action,

$$\frac{(S) \times (E - c)}{c} = K;$$

Rearranging,  $c = E \frac{S}{S + K}$ .

If initial reaction velocity is proportional to  $c$ , initial velocity =  $kE \frac{S}{S + K}$ .

This expression is similar to the dissociation residue curve for an acid  $\left[1 - \alpha = \frac{H}{(H) + K}\right]$  in which we plot the percentage of undissociated acid ( $1 - \alpha$ ) against the negative logarithm of the H ion concentration (pH) and obtain the characteristic S-shaped curve. Similarly, if we plot the initial velocity of inversion of cane sugar against the negative logarithm of the cane sugar concentration for the same concentration of invertase ( $E$ ), we get an S-shaped curve whose mid point gives the constant  $K$ , the dissociation constant for the enzyme-substrate compound.

It should be pointed out that this type of S-shaped curve is also characteristic of Langmuir's adsorption equation for relating the fraction of surface ( $\theta$ ) covered by a mono-layer of gas atoms to the pressure ( $p$ ) of gas. ( $1 - \theta$ ) is the fraction of surface uncovered and the rate of collection of gas =  $k_1 p (1 - \theta)$ . The rate of evaporation of gas from the surface =  $k_2 \theta$ .

At equilibrium,  $k_1 p (1 - \theta) = k_2 \theta$ , and  $\theta = \frac{k_1 p}{k_2 + k_1 p}$ .

The form of the equation is the same and  $\theta$  plotted against the negative logarithm of  $p$  gives the same characteristic S-curve. If we assume that only the substrate adsorbed on the enzyme molecules is activated and undergoes change, the Michaelis-Menten curves can be explained on the Langmuir adsorption isotherm, the mid portion of which corresponds to the Freundlich adsorption relation. The log plots of  $\frac{dx}{dt}$  against time will not give straight lines, since the equilibrium between enzyme and cane sugar adsorbed is continually changing as the cane sugar is converted into glucose and levulose. However the fundamental effect is that the average velocity becomes greater the less the initial cane sugar concentration, as relatively more cane sugar is adsorbed and activated the greater its dilution.

If, on the other hand, the reaction products have the same adsorp-



tion equilibrium as the substrate, the adsorption equilibrium is constant through the reaction and reaction velocity falls off logarithmically as substrate is converted into reaction products. The velocity constant, however, becomes greater the weaker the substrate concentration, corresponding to the relatively greater adsorption from weak solutions. We believe this is the situation in the case of the luciferin-luciferase reaction discussed in this paper.

Another example of the application of the Langmuir adsorption relation to biological phenomena is given by Shoup's (1929) studies on oxygen consumption of luminous bacteria at various oxygen pressures. The curve looks like an adsorption curve and plotted as percentage of maximal respiratory rate (oxygen consumption) against  $-\log$  oxygen pressure gives a characteristic S-shaped curve. The logical explanation would appear to be given by assuming that rate of respiration is determined by the percentage of surface of the respiratory catalysts covered by a layer of oxygen. This should obey the adsorption isotherm.

It is unfortunate that the adsorption and intermediary compound views both lead to the same kinetic picture of enzyme action, for we obviously cannot distinguish between them in this way, as has been pointed out by Hitchcock (1926) in connection with the behavior of proteins.

As a matter of fact these views represent merely our mental picture of what is taking place between minute particles, and we prefer the adsorption explanation which is in line with the recent treatment of contact catalyses (Taylor, 1930; Hinshelwood, 1929).

#### SUMMARY

1. The rapid decay of luminescence in extracts of the ostracod crustacean *Cypridina hilgendorfi*, has been studied by means of a photoelectric-amplifier-string galvanometer recording system.

2. For rapid flashes of luminescence, the decay is logarithmic if ratio of luciferin to luciferase is small; logarithmic plus an initial flash, if ratio of luciferin to luciferase is greater than five. The logarithmic plot of luminescence intensity against time is concave to time axis if ratio of luciferin to luciferase is very large.

3. The velocity constant of rapid flashes of luminescence is approxi-

mately proportional to enzyme concentration, is independent of luciferin concentration, and varies approximately inversely as the square root of the total luciferin (luciferin + oxyluciferin) concentration. For large total luciferin concentrations, the velocity constant is almost independent of the total luciferin.

4. The variation of velocity constant with total luciferin concentration (luciferin + oxyluciferin) and its independence of luciferin concentration is explained by assuming that light intensity is a measure of the luciferin molecules which become activated to oxidize (accompanied with luminescence) by adsorption on luciferase. The adsorption equilibrium is the same for luciferin and oxyluciferin and determines the velocity constant.

We take pleasure in thanking Mr. Charles Butt, Research Assistant in Physiology, for his aid in recording these luminescences.

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## ANNOUNCEMENT

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# DETACHMENT OF BACTERIOPHAGE FROM ITS CARRIER PARTICLES

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The active agent of transmissible lysis of bacteria does not exhibit ~~gradual diminution of its activity on serial dilution but becomes at~~

## CORRECTIONS

In Vol. 13, No. 6, July 20, 1930, page 743, line 24, for "Millon" read "Molisch."

In Vol. 14, No. 2, November 20, 1930, page 283, the last two lines (above footnotes) should read

$$\begin{aligned}\frac{dx}{dt} &= P_K [(H_{ob} - x) (K_{ib} - x) - (K_{ob} + x) (H_{ib} + x)] \\ &= P_K [(H_{ob}K_{ib} - K_{ob}H_{ib}) - x(K_{ib} + H_{ob} + H_{ib} + K_{ob})]\end{aligned}$$

washing of the residual fraction of the active agent with distilled water failed to carry it through the ultrafilter. However, when broth was substituted for water in washing, the active agent reappeared in the ultrafiltrate. In view of our earlier observations concerning the effect of colloids on the filtrability of bacteria,<sup>3</sup> we carried out control experiments, which indicated however that a simple passage of broth through a new membrane similar to that used above apparently failed to increase its permeability. Consequently the effect of broth in causing

passages of phage in the above experiments was interpreted as indicating that the addition of broth to the residue might have caused detachment (elution) of some of the active agent from the coarser particles and its adsorption on and passage with the finer particles of the broth.<sup>2</sup>

If this phenomenon of detachment of the active agent from the carrier particles could be definitely proven, it would supply very weighty evidence against the parasitic nature of bacteriophage. It seemed to us that if, by measuring the particles of the residue on the filter, the particles in the broth, and the particles which go through the filter upon addition of broth to the residue respectively, we found the changes in diffusibility to go hand in hand with changes in the size of carrier particles, then we would be able to verify the detachment of the active agent from its carrier particles. Some time ago we found that the procedure employed by Northrop and Anson<sup>4</sup> for the determination of particle size of carbon monoxide hemoglobin could be used to advantage in measuring the size of particles carrying the phage.<sup>5,6</sup> This procedure consists in determining the rate of diffusion of the unknown substance through porous membranes, the diffusion constants of which were previously determined by measuring the rate of passage through them of such simple substance with known diffusion coefficient as hydrochloric acid.

The diffusion coefficient "*D*" is defined as the quantity of substance that will diffuse across a permeable plane of unit dimensions in unit time under unit concentration gradient or, as calculated by Einstein.<sup>7</sup>

$$(1) \quad D = \frac{dQ}{A \, dt \, \frac{dc}{dx}}$$

By appropriate substitutions Northrop reduces this equation to the following:<sup>4</sup>

$$(2) \quad D = \frac{K \, Q_{cc.} \, Cm.^2}{t \, \text{day}} \text{ in which}$$

*D* = Diffusion coefficient.

*K* = Diffusion constant of the cell.

*Q* = Cc. of substance diffused expressed as a fraction of the original concentration.

*t* = Time in days.

The relation between the diffusion coefficient and the radius of the molecule is found in the following equation devised by Einstein:

$$(3) \quad D = \frac{RT}{N} \cdot \frac{1}{6 \pi r \eta} \text{ in which}$$

$$R = 8.3 \times 10^7 \text{ erg} \times \text{deg.}^{-1} \times \text{mole}^{-1}$$

$$N = 6.06 \times 10^{23} \times \text{mole}^{-1}$$

$$T = 278^\circ \text{ (for } 5^\circ\text{C.)}$$

$$r = \text{Radius of particle including water of hydration in cm.}$$

$$\eta = \text{Viscosity of the solvent (water) at } T$$

$$= 0.01519 \text{ gr. cm.}^{-1} \text{ sec.}^{-1}$$

$$= 0.01519 \text{ erg sec. cm.}^{-1}$$

If water is used as a solvent at  $5^\circ\text{C.}$

$$(4) \quad D = \frac{1.33 \times 10^{-13} \text{ erg deg. mol. cm.}^3}{r \text{ erg deg. mol. cm. sec.}}$$

$$= \frac{1.33 \times 10^{-13} \text{ cm.}^3}{r \text{ sec.}}$$

$$= \frac{1.148 \times 10^{-8} \text{ cm.}^3}{r \text{ day}}$$

$$r = \frac{1.148 \times 10^{-8}}{D}$$

### Apparatus

The cells used in diffusion experiments consisted of thin porous powdered glass discs fused into glass vessels (Empire Laboratory Supply Company Immersion filter No. 8022, size No. 1 or 2, porosity 5 — 7 or < 7). The porous plate of each vessel was filled with rosin and ground down to a thickness of less than .5 mm. The rosin was then removed by chloroform. To the open end of each vessel was attached a piece of heavy walled rubber tubing into which was fitted a glass stop-cock. Three such cells were prepared.\*

### Standardization of Cells

Before using these cells it was necessary to determine the diffusion constant ( $K$ ) for each cell. The cells were thoroughly washed in dichromate cleaning solution and subsequently washed free of acid with distilled water.

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\* During the course of the experiments two of these cells were broken and were replaced by new cells prepared in a similar manner.



During the experiments the cells were held rigid in a vertical position by two adjustable clamps, so that the discs were perfectly horizontal as determined by mercury level.

All operations, while standardizing the cells and later, while determining the diffusion rates in the actual experiments, were conducted in an air chamber kept at 5°C. ( $\pm .5^\circ$ , per 24 hours). All apparatus and solutions were cooled in this air chamber 18–24 hours before they were used.

Exactly 0.1 N HCl was used in the standardization. The cells were washed several times with the HCl and then filled with it. The outside of each cell was then rinsed repeatedly with distilled water and immersed in a beaker containing 30 cc. of distilled water to such a depth that the end of the cell just made contact with the surface of the water without any capillary pull. The beakers, each containing 30 cc. of H<sub>2</sub>O were changed every 10 minutes and the amount of HCl diffused was determined by titration with N/100 NaOH using phenol red as indicator.

TABLE I  
*Diffusion of 0.1 N HCl through the Porous Disks of the Various Cells*

| Cell | Time        | Amount of 0.1 N HCl diffused |
|------|-------------|------------------------------|
|      | <i>days</i> | <i>cc.</i>                   |
| I    | 0.00694     | 0.065                        |
| II   | 0.00694     | 0.013                        |
| III  | 0.00694     | 0.045                        |

This procedure was repeated until titrations indicated that the rate of diffusion of the acid had become approximately constant. This rate for each of the three cells is indicated in Table I. Since the diffusion coefficient of HCl at 5°C. is known to be 1.85 cm.<sup>2</sup> per day,\* therefore, the cell constants as calculated by Equation 2 from the data presented in Table I are:

$$K (\text{Cell I}) = \frac{1.85 \times 0.00694}{0.065} = 0.1975$$

$$K (\text{Cell II}) = \frac{1.85 \times 0.00694}{0.013} = 0.9876$$

$$K (\text{Cell III}) = \frac{1.85 \times 0.00694}{0.045} = 0.2853$$

In making these calculations the amount of acid in the vessel was taken as remaining constant† since it was found in accordance with

\* Extrapolated by Northrop<sup>4</sup> from Ohölin's data.

† The measurements showed that less than 2 per cent of acid diffused during the entire experiment.

earlier observations of Northrop,<sup>4</sup> that the amount of acid diffused was not sufficient to appreciably change the value of "Q".

After each series of determinations in the actual experiments with organic materials, the cells were cleaned in dichromate, washed with distilled water, and sterilized by autoclaving. While cleaning and autoclaving the cells after the first series of determinations two out of three cells used were broken. The remaining cell and two new cells were used in all the subsequent experiments. However from this time on to avoid further accidents the cells were sterilized by immersion into 70 per cent alcohol overnight instead of by autoclaving.

The values of  $K$  for the cells at this time were found to be:

$$K \text{ (Cell I) } = 0.2038$$

$$K \text{ (Cell IV) } = 0.1712$$

$$K \text{ (Cell V) } = 0.2140$$

Frequent subsequent determinations of the values of  $K$  for the cells showed that these values did not change during the remaining experiments.

## EXPERIMENTAL

### (a) *Preparation of Materials to be Tested*

In the experiments outlined below we have compared, by the procedure just described, the diffusion coefficients of the following preparations of phage (Table VI).

*Test Solution "A". Crude Broth Filtrate.*—This consisted of a culture of *B. coli* in 1000 cc. of plain extract broth to which "BW" bacteriophage was added to cause complete lysis of bacteria. The lysed culture was filtered through a Berkefeld (N) filter and thus represented an ordinary crude lytic filtrate. The titration of this filtrate by the tenfold dilution method of Appelmans<sup>3</sup> indicated a phage titer of  $10^{-9}$  cc. 1 cc. of this filtrate was so diluted with sterile broth of the same lot as to bring its titer to  $10^{-4}$  cc. and in this condition it was used for determining its diffusion coefficient.

*Test Solution "B". Ultrafiltered Phage.*—Another portion (250 cc.) of the undiluted crude filtrate was subjected to ultrafiltration under a pressure of about 700 mm. of mercury, through a sterile 7 per cent collodion membrane deposited on the walls of an alundum thimble of 100 cc. capacity.\* The titer of bacteriophage in the ultrafiltrate thus obtained was found to be  $10^{-5}$  cc. Of this ultrafiltrate,

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\* The exact procedure used in the preparation of this membrane was described by us earlier.<sup>2</sup>

25 cc. were diluted with 225 cc. of broth previously subjected to ultrafiltration through a similar 7 per cent collodion membrane. This diluted ultrafiltrate was found to have a phage titer of  $10^{-4}$  cc. and in this condition was used for determination of its diffusion coefficient.

*Test Solution "C". Washings from Residue Suspended in Water.*—The residue remaining in the ultrafilter in the preparation of the ultrafiltrate "B" was repeatedly washed by diluting it with 100 cc. portions of sterile distilled water under a pressure of 700 mm. of mercury. As each new portion of water was placed into the thimble, the surface of collodion was gently swabbed with a sterile cotton swab to secure a more thorough washing. Each 100 cc. portion of the fluid which filtered through was collected into a separate sterile filter flask and titrated for its phage content. This procedure was repeated in all eight times and the phage titers of respective filtrates obtained were:  $10^{-4}$  cc.;  $10^{-4}$  cc.;  $10^{-4}$  cc.;  $10^{-3}$  cc.;  $10^{-3}$  cc.;  $10^{-3}$  cc.;  $10^{-2}$  cc.;  $10^{-2}$  cc. The first two filtrates were discarded and the subsequent six filtrates were mixed and the resulting mixture which was found to have a phage titer of  $10^{-4}$  cc. was used for determining the diffusion coefficient.

*Test Solution "D". Washed Residue Suspended in Water.*—The washed residue now remaining on the collodion membrane was suspended in 40 cc. of sterile distilled water. To remove any portion of the residue which may have adhered to the membrane a sterile cotton swab was employed. The suspension of the residue thus secured was found to have a phage titer of  $10^{-9}$  cc. A 0.1 cc. portion of this washed residue suspension was removed and so *diluted with sterile distilled water* as to give a phage titer of  $10^{-5}$  cc.\* This diluted suspension was used for determining the diffusion coefficient.

*Test Solution "E". Ultrafiltrate from Residue Suspended in Diluted Ultrafiltered Broth.*—Another portion (10 cc.) of the above washed residue suspension (phage titer  $10^{-9}$  cc.) was *diluted with 30 cc. of ultrafiltered broth* (pH = 7.2). The mixture was thoroughly shaken and after standing for 1 hour was further diluted with sterile distilled water to a volume of 250 cc., thoroughly mixed, and immediately subjected to ultrafiltration through the same 7 per cent membrane as had been used in the preceding experiments. The ultrafiltrate was collected under a pressure of 700 mm. of mercury and was found to have a phage titer of  $10^{-4}$  cc. The diffusion coefficient of this ultrafiltrate was determined.

### (b) Diffusion Experiments

Diffusion cells prepared and standardized as described above were washed several times with the phage solution and then filled with it. The outside of the

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\* The titer of this solution was made  $10^{-5}$  cc. instead of  $10^{-4}$  cc. as in the case of other solutions because preliminary experiments had shown that due to the relatively large size of particles present in this solution, the rate of diffusion was too slow to be measured accurately in the 30 minute period allowed for diffusion in these experiments.

cells was rinsed with a liquid of the same composition as that in the cells but free from phage. 30 cc. of the same liquid were placed into a sterile beaker and the diffusion cells were dipped into this liquid exactly as described above in the experiments with HCl. The diffusion of the phage was continued for 30 minutes and at the end of this time the amount of phage diffused was determined by titration of the outer liquid. Sixteen consecutive 30 cc. samples of diffusate were obtained from each cell. It was found by titration of these that the rate of diffusion becomes approximately constant by the end of the second hour. For this reason the phage titer of the first four samples were not considered and only the results of titration of the remaining twelve samples were used in calculating the average titer of the diffusate.\*

To minimize the experimental error, the rate of diffusion of each test solution was measured by means of each one of the three cells and the entire determination was repeated at least twice. Thus in each instance the figures indicating the total number of phage units present in the diffusates (Table VI, Col. 5) used in the subsequent calculation of diffusion rates (Table VI, Col. 9) represented an average of at least six separate determinations in each of which the titer represented an average of titrations of twelve consecutive portions of diffusate.

### (c) *Titration of Diffusates*

To determine the total number of units of phage present in each 30 cc. sample of diffusate (see Table VI, Col. 5) each solution was thoroughly mixed by repeated aspiration with a pipette and then increasing amounts of this solution were placed in tubes (see Table II).

Since many of these solutions were highly diluted with distilled water, whenever necessary sufficient amounts of 5 per cent broth were added to each tube to bring the final concentration of broth in all tubes to 1 per cent. The total volume in each tube was then made up to 10 cc. by the addition of 1 per cent broth and the contents were inoculated with 0.1 cc. of a suspension of *B. coli* containing about  $1 \times 10^9$  of organisms per cc. Preliminary readings were made at the end of 24 and 48 hours of incubation at which time final transfers were made and the results read after 4 and again after 18 hours.<sup>1</sup> The amount of test solution present in the last tube of the uninterrupted series showing lysis was taken as indicating the presence of one unit of phage. The titration of Test Solution E is given below in detail as an illustration of this procedure.

*Example of Titration of Test Solution E.*—Sterile diffusion cells (I, IV, and V)

\* Before beginning the actual experiments each test solution was subjected to a preliminary diffusion test using one cell. This was made in order to locate the approximate range in which the titrations of diffusates will fall and where necessary the test solutions were so diluted as to bring the titrations in all cases within a comparatively narrow range and thus facilitate the carrying out of final experiments.

were filled with Test Solution "E" and the outside surface was repeatedly washed with a liquid consisting of twelve volumes of ultrafiltered broth and eighty-eight volumes of distilled water. The diffusion cells were leveled and dipped into beakers each containing 30 cc. of the same liquid. Diffusion was allowed to proceed for 30 minutes, at which time the beakers were removed for titration and another set of beakers with fresh solution was substituted. This procedure was repeated for sixteen consecutive periods of 30 minutes each. The first four diffusates obtained from each of the three diffusion cells were discarded and the remain-

TABLE II  
*Detail of the Titration of Diffusates of Cell I\**

| Tube number.....               | 1 | 2   | 3   | 4   | 5   | 6   | 7   | 8   | 9   | 10  | Volume of diffusate containing 1 unit of phage |
|--------------------------------|---|-----|-----|-----|-----|-----|-----|-----|-----|-----|--|
| Amount of diffusate in cc..... | 1 | 0.9 | 0.8 | 0.7 | 0.6 | 0.5 | 0.4 | 0.3 | 0.2 | 0.1 |  |
| Time of sampling               |   |     |     |     |     |     |     |     |     |     |  |
| hrs.                           |   |     |     |     |     |     |     |     |     |     | cc.  |
| 2.5                            | + | +   | +   | +   | +   | +   | +   | -   | -   | -   | 0.4  |
| 3.0                            | + | +   | +   | +   | +   | +   | -   | -   | -   | -   | 0.5  |
| 3.5                            | + | +   | +   | +   | +   | +   | -   | -   | -   | -   | 0.5  |
| 4.0                            | + | +   | +   | +   | +   | +   | +   | +   | -   | -   | 0.3  |
| 4.5                            | + | +   | +   | +   | +   | +   | +   | -   | -   | -   | 0.4  |
| 5.0                            | + | +   | +   | +   | +   | +   | +   | -   | -   | -   | 0.4  |
| 5.5                            | + | +   | +   | +   | +   | +   | +   | +   | -   | -   | 0.3  |
| 6.0                            | + | +   | +   | +   | +   | +   | +   | +   | -   | -   | 0.3  |
| 6.5                            | + | +   | +   | +   | +   | +   | -   | -   | -   | -   | 0.5  |
| 7.0                            | + | +   | +   | +   | +   | +   | +   | -   | -   | -   | 0.4  |
| 7.5                            | + | +   | +   | +   | +   | +   | +   | -   | -   | -   | 0.4  |
| 8.0                            | + | +   | +   | +   | +   | +   | +   | -   | -   | -   | 0.4  |
| Average.....                   |   |     |     |     |     |     |     |     |     |     | 0.4  |

\* + = lysis.

- = no lysis.

ing twelve diffusates were titrated as shown in Table II. The data recorded in this table were obtained from titration of diffusates of Cell I. Diffusates from Cells IV and V were titrated in a similar manner, and the results of these titrations are recorded in Table III.

As was to be expected, the indicating tube was not the same in all twelve titrations of diffusate (Table II), because of the fact that bacteriophage is distributed in the medium in the form of particles as has been fully discussed in an earlier paper.<sup>1</sup> It is for this reason that for calculations an average of twelve

titrations was used. The limits of error which may arise from such a procedure, however, would account only for a fraction of difference in the diffusion coefficients

TABLE III  
*Complete Results of Titration of Diffusates of Test Solution "E"*

| 1st Experiment                        |  |         |        | 2nd Experiment   |         |        |
|---------------------------------------|--|---------|--------|--|---------|--------|
| Time of sampling<br><i>hrs.</i>       | Amounts of diffusates containing 1 unit<br>of phage in cc. |         |        | Amounts of diffusates containing 1 unit<br>of phage in cc. |         |        |
|                                       | Cell I   | Cell IV | Cell V | Cell I   | Cell IV | Cell V |
| 2.5                                   | 0.4  | 0.3     | 0.5    | 0.4  | 0.2     | 0.3    |
| 3.0                                   | 0.5  | 0.2     | 0.5    | 0.4  | 0.4     | 0.3    |
| 3.5                                   | 0.5  | 0.3     | 0.3    | 0.3  | 0.2     | 0.4    |
| 4.0                                   | 0.3  | 0.4     | 0.4    | 0.4  | 0.3     | 0.4    |
| 4.5                                   | 0.4  | 0.2     | 0.3    | 0.5  | 0.4     | 0.3    |
| 5.0                                   | 0.4  | 0.3     | 0.5    | 0.3  | 0.4     | 0.5    |
| 5.5                                   | 0.3  | 0.3     | 0.4    | 0.4  | 0.3     | 0.4    |
| 6.0                                   | 0.3  | 0.4     | 0.4    | 0.5  | 0.2     | 0.5    |
| 6.5                                   | 0.5  | 0.3     | 0.3    | 0.3  | 0.4     | 0.4    |
| 7.0                                   | 0.4  | 0.4     | 0.4    | 0.4  | 0.4     | 0.5    |
| 7.5                                   | 0.4  | 0.4     | 0.5    | 0.4  | 0.4     | 0.3    |
| 8.0                                   | 0.4  | 0.3     | 0.4    | 0.3  | 0.3     | 0.4    |
| Average of twelve<br>titrations ..... | 0.4  | 0.32    | 0.41   | 0.38   | 0.33    | 0.39   |

TABLE IV  
*Preliminary Titration of Test Solution "E" (in Sextuplicate)\**

| Tube number.....                                      |   | 1             | 2             | 3             | 4             | 5             |
|---|---|---------------|---------------|---------------|---------------|---------------|
| Total amount of test solution<br>E in each tube. .... |   | $10^{-1}$ cc. | $10^{-2}$ cc. | $10^{-3}$ cc. | $10^{-4}$ cc. | $10^{-5}$ cc. |
| Amount of test solution E in<br>each cc.....          |   | $10^{-1}$ cc. | $10^{-2}$ cc. | $10^{-3}$ cc. | $10^{-4}$ cc. | $10^{-5}$ cc. |
| Repeated ti-<br>trations                              | a | +             | +             | +             | +             | —             |
|   | b | +             | +             | +             | +             | —             |
|   | c | +             | +             | +             | —             | —             |
|   | d | +             | +             | +             | +             | —             |
|   | e | +             | +             | +             | +             | —             |
|   | f | +             | +             | +             | +             | —             |

\* + = lysis.

— = no lysis.

actually observed in comparing the test solutions (B), (C), (D), and (E) (See Table VI) as the calculations on one of the substrata (E) will show.

For Cell I (whose constant  $K = 0.2038$ ) the average amount of diffusate containing 1 unit of phage was found to be 0.4 cc. (Table II) or in each 30 cc. sample of this diffusate there were 75 units of phage. Since, as will be shown later (Table VI, Col. 6), 1 cc. of original Solution E contains 25,000 phage units, the "Q" in this instance is  $75/25,000 = 0.003$ .

TABLE V  
*Final Titration of Test Solution E (in Sextuplicate)*

| Tube number.....                        |   | 1             | 2                    | 3                    | 4                    | 5                    | 6                    | 7                    | 8                    | 9                    | 10        |
|---|---|---------------|----------------------|----------------------|----------------------|----------------------|----------------------|----------------------|----------------------|----------------------|-----------|
| Amount of test solution E in cc.....    |   | 1 cc.         | 0.9 cc.              | 0.8 cc.              | 0.7 cc.              | 0.6 cc.              | 0.5 cc.              | 0.4 cc.              | 0.3 cc.              | 0.2 cc.              | 0.1 cc.   |
| Absolute amount of solution E in cc.... |   | $10^{-4}$ cc. | $0.9 \times 10^{-4}$ | $0.8 \times 10^{-4}$ | $0.7 \times 10^{-4}$ | $0.6 \times 10^{-4}$ | $0.5 \times 10^{-4}$ | $0.4 \times 10^{-4}$ | $0.3 \times 10^{-4}$ | $0.2 \times 10^{-4}$ | $10^{-5}$ |
| Repeated titra-<br>tions                | a | +             | +                    | +                    | +                    | +                    | +                    | +                    | -                    | -                    | -         |
|   | b | +             | +                    | +                    | +                    | +                    | +                    | -                    | -                    | -                    | -         |
|   | c | +             | +                    | +                    | +                    | +                    | +                    | +                    | -                    | -                    | -         |
|   | d | +             | +                    | +                    | +                    | +                    | +                    | +                    | -                    | -                    | -         |
|   | e | +             | +                    | +                    | +                    | +                    | +                    | +                    | +                    | -                    | -         |
|   | f | +             | +                    | +                    | +                    | +                    | +                    | +                    | -                    | -                    | -         |

(a) Using this value in equation (2), the diffusion coefficient of this solution will be:

$$D = \frac{K Q \text{ cc. cm.}^2}{t \text{ day}} = \frac{0.2038 \times 0.003}{0.0208} = 0.0294 \text{ cm.}^2 \text{ per day.}$$

(b) Should the volume of diffusate containing 1 unit of phage have been 0.3 cc. instead of 0.4 cc., each 30 cc. sample would have contained 100 units and consequently "Q" would have been  $100/25,000 = 0.004$  and the diffusion coefficient would have been

$$D = \frac{0.2038 \times 0.004}{0.0208} = 0.0392 \text{ cm.}^2 \text{ per day.}$$

TABLE VI  
Results of Diffusion Experiments

| 1             | 2            | 3           | 4                    | 5   | 6  | 7   | 8                   | 9   | 10   | 11   |
|---------------|--------------|-------------|----------------------|---|--|---|---------------------|---|--|--|
| Test solution | Trial number | Cell number | Cell constant<br>(K) | Total units of phage in 30 cc. of diffusate | Units of phage in 1 cc. of test solution | Fraction of phage diffused<br>Column 5<br>Column 6<br>(Q) | Time<br>(t)<br>days | Diffusion rate as calculated by equation (2)<br>(D) | Average diffusion rate of test solution<br>(Average D) | Average radius of particle as calculated by equation (4) in millimicrons |
| A             | 1            | I           | 0.2038               | 88  | 33,300                                   | 0.00264   | 0.0208              | 0.02586   | 0.02583  | 4.4  |
|               | 1            | IV          | 0.1712               | 100   | "  | 0.00300   | "                   | 0.02469   |  |  |
|               | 1            | V           | 0.2140               | 86  | "  | 0.00258   | "                   | 0.02654   |  |  |
|               | 2            | I           | 0.2038               | 88  | "  | 0.00264   | "                   | 0.02586   |  |  |
|               | 2            | IV          | 0.1712               | 103   | "  | 0.00310   | "                   | 0.02551   |  |  |
|               | 2            | V           | 0.2140               | 86  | "  | 0.00258   | "                   | 0.02654   |  |  |
| B             | 1            | I           | 0.2038               | 100   | 12,500                                   | 0.00800   | 0.0208              | 0.0784  | 0.0811   | 1.4  |
|               | 1            | IV          | 0.1712               | 136   | "  | 0.01090   | "                   | 0.0895  |  |  |
|               | 1            | V           | 0.2140               | 94  | "  | 0.00752   | "                   | 0.0774  |  |  |
|               | 2            | I           | 0.2038               | 97  | "  | 0.00776   | "                   | 0.0760  |  |  |
|               | 2            | IV          | 0.1712               | 120   | "  | 0.00960   | "                   | 0.0790  |  |  |
|               | 2            | V           | 0.2140               | 104   | "  | 0.00832   | "                   | 0.0856  |  |  |
| C             | 1            | I           | 0.1975               | 205   | 10,000                                   | 0.0205  | 0.0208              | 0.1948  | 0.1923   | 0.6  |
|               | 1            | II          | 0.9876               | 41  | "  | 0.0041  | "                   | 0.1946  |  |  |
|               | 1            | III         | 0.2853               | 133   | "  | 0.0133  | "                   | 0.1820  |  |  |
|               | 2            | I           | 0.1975               | 200   | "  | 0.0200  | "                   | 0.1943  |  |  |
|               | 2            | II          | 0.9876               | 40  | "  | 0.0040  | "                   | 0.1875  |  |  |
|               | 2            | III         | 0.2853               | 150   | "  | 0.0150  | "                   | 0.2010  |  |  |
| D             | 1            | I           | 0.2038               | 100   | 100,000                                  | 0.00100   | 0.0208              | 0.009798  | 0.01003  | 11.4   |
|               | 1            | IV          | 0.1712               | 120   | "  | 0.00120   | "                   | 0.009876  |  |  |
|               | 1            | V           | 0.2140               | 97  | "  | 0.00097   | "                   | 0.009998  |  |  |
|               | 2            | I           | 0.2038               | 103   | "  | 0.00103   | "                   | 0.010090  |  |  |
|               | 2            | IV          | 0.1712               | 120   | "  | 0.00120   | "                   | 0.009876  |  |  |
|               | 2            | V           | 0.2140               | 103   | "  | 0.00103   | "                   | 0.010600  |  |  |
| E             | 1            | I           | 0.2038               | 75  | 25,000                                   | 0.00300   | 0.0208              | 0.0294  | 0.0305   | 4.0  |
|               | 1            | IV          | 0.1712               | 94  | "  | 0.00376   | "                   | 0.0309  |  |  |
|               | 1            | V           | 0.2140               | 73  | "  | 0.00296   | "                   | 0.0302  |  |  |
|               | 2            | I           | 0.2038               | 79  | "  | 0.00316   | "                   | 0.0309  |  |  |
|               | 2            | IV          | 0.1712               | 91  | "  | 0.00364   | "                   | 0.0299  |  |  |
|               | 2            | V           | 0.2140               | 77  | "  | 0.00308   | "                   | 0.0316  |  |  |





TABLE VI  
Results of Diffusion Experiments

| 1             | 2            | 3           | 4                    | 5   | 6  | 7   | 8                   | 9   | 10   | 11   |
|---------------|--------------|-------------|----------------------|---|--|---|---------------------|---|--|--|
| Test solution | Trial number | Cell number | Cell constant<br>(K) | Total units of phage in 30 cc. of diffusate | Units of phage in 1 cc. of test solution | Fraction of phage diffused<br>Column 5<br>Column 6<br>(Q) | Time<br>(t)<br>days | Diffusion rate as calculated by equation (2)<br>(D) | Average diffusion rate of test solution<br>(Average D) | Average radius of particle as calculated by equation (4) in millimicrons |
| A             | 1            | I           | 0.2038               | 88  | 33,300                                   | 0.00264   | 0.0208              | 0.02586   | 0.02583  | 4.4  |
|               | 1            | IV          | 0.1712               | 100   | "  | 0.00300   | "                   | 0.02469   |  |  |
|               | 1            | V           | 0.2140               | 86  | "  | 0.00258   | "                   | 0.02654   |  |  |
|               | 2            | I           | 0.2038               | 88  | "  | 0.00264   | "                   | 0.02586   |  |  |
|               | 2            | IV          | 0.1712               | 103   | "  | 0.00310   | "                   | 0.02551   |  |  |
|               | 2            | V           | 0.2140               | 86  | "  | 0.00258   | "                   | 0.02654   |  |  |
| B             | 1            | I           | 0.2038               | 100   | 12,500                                   | 0.00800   | 0.0208              | 0.0784  | 0.0811   | 1.4  |
|               | 1            | IV          | 0.1712               | 136   | "  | 0.01090   | "                   | 0.0895  |  |  |
|               | 1            | V           | 0.2140               | 94  | "  | 0.00752   | "                   | 0.0774  |  |  |
|               | 2            | I           | 0.2038               | 97  | "  | 0.00776   | "                   | 0.0760  |  |  |
|               | 2            | IV          | 0.1712               | 120   | "  | 0.00960   | "                   | 0.0790  |  |  |
|               | 2            | V           | 0.2140               | 104   | "  | 0.00832   | "                   | 0.0856  |  |  |
| C             | 1            | I           | 0.1975               | 205   | 10,000                                   | 0.0205  | 0.0208              | 0.1948  | 0.1923   | 0.6  |
|               | 1            | II          | 0.9876               | 41  | "  | 0.0041  | "                   | 0.1946  |  |  |
|               | 1            | III         | 0.2853               | 133   | "  | 0.0133  | "                   | 0.1820  |  |  |
|               | 2            | I           | 0.1975               | 200   | "  | 0.0200  | "                   | 0.1943  |  |  |
|               | 2            | II          | 0.9876               | 40  | "  | 0.0040  | "                   | 0.1875  |  |  |
|               | 2            | III         | 0.2853               | 150   | "  | 0.0150  | "                   | 0.2010  |  |  |
| D             | 1            | I           | 0.2038               | 100   | 100,000                                  | 0.00100   | 0.0208              | 0.009798  | 0.01003  | 11.4   |
|               | 1            | IV          | 0.1712               | 120   | "  | 0.00120   | "                   | 0.009876  |  |  |
|               | 1            | V           | 0.2140               | 97  | "  | 0.00097   | "                   | 0.009998  |  |  |
|               | 2            | I           | 0.2038               | 103   | "  | 0.00103   | "                   | 0.010090  |  |  |
|               | 2            | IV          | 0.1712               | 120   | "  | 0.00120   | "                   | 0.009876  |  |  |
|               | 2            | V           | 0.2140               | 103   | "  | 0.00103   | "                   | 0.010600  |  |  |
| E             | 1            | I           | 0.2038               | 75  | 25,000                                   | 0.00300   | 0.0208              | 0.0294  | 0.0305   | 4.0  |
|               | 1            | IV          | 0.1712               | 94  | "  | 0.00376   | "                   | 0.0309  |  |  |
|               | 1            | V           | 0.2140               | 73  | "  | 0.00296   | "                   | 0.0302  |  |  |
|               | 2            | I           | 0.2038               | 79  | "  | 0.00316   | "                   | 0.0309  |  |  |
|               | 2            | IV          | 0.1712               | 91  | "  | 0.00364   | "                   | 0.0299  |  |  |
|               | 2            | V           | 0.2140               | 77  | "  | 0.00308   | "                   | 0.0316  |  |  |



ing the diffusion rates of each of the test solutions. The average diffusion rates thus obtained in each case were substituted for  $D$  in equation (4) which now could be solved and thus the average radius of particles present in each test solution determined (Table VI, Column 11).

These figures indicate that the fraction which passes freely through 7 per cent acetic collodion membrane (Test Solution B) contains particles approaching in the average  $1.4 \text{ m}\mu$  in radius, whereas the fraction retained by the ultrafilter (Test Solution D) consists of particles measuring in the average  $11.4 \text{ m}\mu$  in radius. This residual fraction still contained a certain proportion of small particles, and when it was washed repeatedly with water, the particles thus carried through the ultrafilter (Test Solution C) measured in the average  $0.6 \text{ m}\mu$  in radius. However, when the residue held by the membrane was suspended in ultrafiltered broth the fluid which passed the filter (Test Solution E) contained particles with the average radius size of  $4.0 \text{ m}\mu$  or nearly the size of particles present in the original broth filtrate (Test Solution A) the radius of which approached the average of  $4.4 \text{ m}\mu$ .

#### DISCUSSION

On the basis of our earlier findings concerning the distribution of bacteriophage during dialysis or as result of ultrafiltration we have suggested that the particles carrying the active agent are not autonomous quanta of the phage, but represent colloidal particles of the medium which carry the active agent by adsorption.<sup>2</sup> Moreover, these results suggested that these particles apparently vary in size within rather broad limits. The findings reported in this paper substantiate this interpretation. We find that while the average size of particles carrying phage in an ordinary lytic broth filtrate approximate  $4.4 \text{ m}\mu$  in radius, it is possible to separate by ultrafiltration fractions consisting of particles varying in size from  $0.6 \text{ m}\mu$  to  $11.4 \text{ m}\mu$ . Of course these figures do not represent the extreme limits of variation in the size of particles carrying phage. Our experience indicates that average size of phage-carrying particles varies considerably in different batches.\* Moreover, it is evident that by the use of less permeable

\* The average size of phage carrying particles in an ordinary lytic filtrate varies from one batch to another depending probably on the degree of disintegration of

membranes, particles of smaller radius than  $0.6\text{ m}\mu$  can be obtained. On the other hand, by forcing the suspension of the residue (test solution D) with active particles of  $11.4\text{ m}\mu$  in radius on the average through membranes more permeable than those we have used (7 per cent acetic acid collodion), one may undoubtedly obtain a fraction composed of particles of considerably larger dimensions.

The accuracy of numerical values secured by us in calculating the radius of particles in the different fractions depends of course on the validity of the procedure employed by us in these determinations.\* The formulae used in the calculations hold only for homogeneous suspensions. Under the conditions of our experiments the homogeneity of different fractions was not ascertained directly. The presence of particles of various sizes in the suspension, would lead to too small values for the diffusion coefficient, since the small particles will diffuse out first and the quantity of small particles remaining will be less than the total number as determined by titration. This source of error, therefore, would tend to make the calculated radii too large. Since the procedure yielded surprisingly reproducible results on repetition the suspensions, if not uniform, were always of nearly the same composition.

These experiments showed that in the particular batch of crude lytic filtrate, the particles which carried lytic properties approximated  $4.4\text{ m}\mu$  in radius (Table VI, Test Solution A). When this phage containing broth was forced through 7 per cent acetic collodion, the liquid which passed through the membrane contained active particles  $1.4\text{ m}\mu$  in radius on the average. This ultrafiltrate represented only a fraction of the lytic agent originally present in the broth. The re-

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the bacterial residue after lysis, as well as on the texture of a particular filter candle. Thus for example in an earlier series of experiments similar to those we are now reporting we have found the average size of particles  $6\text{ m}\mu$  in radius with the corresponding fractions of  $2.2\text{ m}\mu$  and  $14\text{ m}\mu$  respectively (Bronfenbrenner and Hetler, *Proc. Soc. Exp. Biol. and Med.*, 1930, 27, 263).

\* It is interesting to note, however, that our figures fall within the extreme values reported by other investigators who used different methods for measuring the size of phage particles. In their calculations only crude unfractionated phage was used and results secured by different individuals varied from  $5\text{ m}\mu$  up to  $120\text{ m}\mu$  as the diameter of the phage particle.<sup>9,10</sup>

mainder of the active substance was retained by the membrane, presumably because it was adsorbed on particles of such a magnitude that they were unable to pass through the pores of the membrane. This residue, however, was not composed entirely of large particles. A certain number of small particles remained with it caught between the larger ones. When the residue was resuspended in water and then forced through the membrane the water which came through contained these small particles (Table VI, Test Solution C) which measured on the average  $0.6 \text{ m}\mu$  in radius. After thorough washing the particles of the residue were suspended in water (Table VI, Test Solution D) and proved to be approximately  $11.4 \text{ m}\mu$  in radius. However, when another portion of the same washed residue (radius  $11.4 \text{ m}\mu$ ) was suspended in broth (from which coarser particles were removed by preliminary ultrafiltration), and this suspension was forced through the same membrane, the particles which now appeared in the ultrafiltrate (Table VI, Test Solution E) measured  $4.0 \text{ m}\mu$ .

Provided, as we have shown earlier,<sup>2</sup> that the permeability of the membrane was not appreciably affected by the presence of broth,\* this finding suggests that some of the active substance was detached from the larger particles ( $11.4 \text{ m}\mu$ ) and was carried through the membrane adsorbed on the smaller ( $4.0 \text{ m}\mu$ ) colloidal particles present in the broth.

### CONCLUSIONS

The active substance (phage) present in the lytic broth filtrate is distributed through the medium in the form of particles. These particles vary in size within broad limits. The average size of these particles as calculated on the basis of the rate of diffusion approximates  $4.4 \text{ m}\mu$  in radius. Fractionation by means of ultrafiltration permits partial separation of particles of different sizes. Under conditions of experiments here reported the particles varied in the radius size from  $0.6 \text{ m}\mu$  to  $11.4 \text{ m}\mu$ . The active agent apparently is not intimately

\* That the presence of the broth was not responsible for increase in the permeability of the membrane can be concluded also from the fact that in the process of preparation of Test Solution B all the particles carrying phage were suspended in broth, and yet the larger particles must have been kept back, since the average size of the particles in the ultrafiltrate was found to be only  $1.4 \text{ m}\mu$  in diameter.

identified with these particles. It is merely carried by them by adsorption, and under suitable experimental conditions it can be detached from the larger particles and redistributed on smaller particles of the medium.

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buffer solutions flowing through quartz diaphragms covered with sorbed crude egg albumin. He employed this system in order to compare his values of  $\zeta_s$  with values of  $\zeta_e$  obtained by Abraham and by Freundlich and Abramson.<sup>4</sup> The agreement between  $\zeta_s$  and  $\zeta_e$  found by Briggs was indeed quite remarkable, as Goettner points out, and substantiated quite well the underlying soundness of the present theory. This result had been predicted by one of us on the basis of experiments with protein-covered quartz particles; it was shown that, since quartz particles covered with egg albumin have the same mobility regardless of size and shape, if the theory is correct the streaming potentials across diaphragms composed of similar particles should give a ratio of  $\frac{\zeta_e}{\zeta_s} = 1.00$ , approximately.

Briggs' data on streaming potentials were obtained in 0.001 M mixtures of HCl-LiCl, while the values of  $\zeta_e$  had been calculated from data obtained in N/50 acetate buffers. In consequence, a strict quantitative comparison of  $\zeta_s$  and  $\zeta_e$  was hindered by the fact that important differences in ionic strength and ionic species were present in the two series of experiments. It is necessary at this point to stress the significance of data previously obtained<sup>7</sup> which describe the relationship between electrophoretic and electroosmotic mobilities; it has been found that the ratio of  $\zeta_e$  to  $\zeta_s$ , the potentials calculated on the basis of electroosmotic experiments for *flat* and for *curved surfaces*, respectively, covered with protein, is 1.00, very nearly. This ratio is not dependent upon the bulk constitution of the particle. Since, therefore,  $\zeta_e$  and  $\zeta_s$  are very intimately connected, on the basis of the present experiments it is evident that a similar relationship should exist between  $\zeta_e$  and  $\zeta_s$ . Whatever may be the theoretical justification for believing that electrophoresis and electroosmosis differ in some fundamental way, the experiments just cited certainly give excellent evidence that these differences, if they do exist, are probably within the limits of error of the technic employed. Thon,<sup>8</sup> for example, recently pointed out that the positions of the well known maxima and minima in the  $\zeta$ -concentration curves differ in electrophoresis and streaming potential measurements on substances having the same constitution. It will be evident from the following discussion that the simple comparison of the sort made by Thon is not a sufficient test

the theory postulating agreement between the values of  $\zeta_c$ ,  $\zeta_s$ , and  $\zeta_e$ ; for surfaces can be employed to test the electrokinetic theory under discussion only when *surface properties* of the materials studied are the same. This is discussed in detail in the following paragraphs.

The experimental problem, in reality, may be said to center about this question: What surfaces are most suitable for the determination of the relationships between  $\zeta_c$ ,  $\zeta_s$ , and  $\zeta_e$ ? The answer to this query must concern itself with the following facts:

1. It is evident from equation (1) that the velocity,  $V$ , depends upon the potential,  $\zeta$ . If we consider the double layer as a rigid system in the Helmholtz sense,

$$\zeta = \frac{4\pi\sigma}{D}\lambda, \quad (3)$$

where  $\lambda$  is the thickness of the double layer,  $\sigma$  being the density of charge. The potential,  $\zeta$ , and in consequence the velocity,  $V$ , can vary with the chemical constitution of the surface, which can determine  $\sigma$ , and with the thickness of the double layer  $\lambda$ . As Müller<sup>9</sup> has emphasized, the theory of Debye and Hückel leads to the relationship,

$$\lambda = \frac{1}{\kappa} \cdot \frac{\kappa R}{1 + \kappa R}, \quad (4)$$

where  $R$  is the radius of the particle (and here the radius of curvature of all points on the surface),  $\mu = \frac{1}{2}\sum n_i z_i^2$ , one half the sum of the products of the concentrations of all ions of the  $i^{\text{th}}$  type into the squares of their valences;  $\kappa = 0.3\sqrt{\mu}10^8$ . The thickness of the double layer is independent of  $R$  within the limits of experimental error when  $\kappa R \gg 1$ . This is evident from (4), for then

$$\lambda = \frac{1}{\kappa}, \text{ very nearly;}$$

and if the chemical make-up of the surface does not appreciably change with change in  $R$ , there results the familiar consequence of the Helmholtz theory in equation (1); this, lacking a size term, predicts  $V$  to be independent of particle radius.

2. There are two groups of relevant experimental data<sup>10</sup> available for the testing of theories of electrokinetic phenomena. One group

concerns microscopic particles whose electrical mobilities are independent of size and shape. This group is characterically represented by quartz particles and paraffin oil droplets, each covered with adsorbed protein. The other group comprises mainly emulsions of the type studied by Mooney.<sup>11</sup> This author found, for example, that the droplets of an emulsion prepared by shaking a *mixture* of red oil and benzyl chloride in distilled water, have mobilities which depend upon  $R$ . Up to  $R = 0.07$  mm., approximately, the mobility increases rapidly with increasing radius. Droplets from 0.07 to 0.22 mm. in radius show no differences in mobility within the limits of experimental error. Mooney's interpretation of his data is slightly different from ours.

3. The group of particles whose mobilities depend upon  $R$  is not suitable for the investigation of the relationship between  $\zeta_e$ ,  $\zeta_s$ , and  $\zeta_o$ , for then the ratios obtained depend upon  $R$ . In remarkable contrast is the behavior of protein-covered particles. These particles are unaffected by slight changes in ionic strength and ionic type (with the exception of the hydrogen ion activity), in the case of ions of low valence. Because in the case of certain microscopic particles, regardless of size, shape, and bulk conductance, all particles have equal mobilities, and because little if any difference is to be found in the mobilities of adsorbed ( $R = 10^{-4}$  cm.) and freely-dispersed protein ( $R = 10^{-7}$  cm.), it seems logical to assume that a comparison of  $\zeta_e$ ,  $\zeta_s$ , and  $\zeta_o$  is most suitably carried out with these systems, which for practical purposes have mobilities independent of  $R$ .\*

$\zeta_e$  has now been reinvestigated in electrophoresis apparatus which has enhanced the accuracy of this method. Solutions of the same ionic strengths and ionic species as those used by Briggs have been employed. The agreement found here for  $\zeta_e$  and  $\zeta_s$  is not as satisfactory as that deduced from older experiments. A suitable explanation is proposed.

\* Recent preliminary experiments on quartz particles covered with adsorbed crystalline serum albumin have confirmed earlier data.<sup>12</sup> The mobilities of these quartz particles were very close to the mobilities observed under similar conditions by Tiselius.<sup>13</sup> These and related data will be presented in a further communication.

### Methods

During the past 3 years modifications of the technic for measuring electrical mobilities have been developed. It is desirable to describe and to reemphasize these at this time.

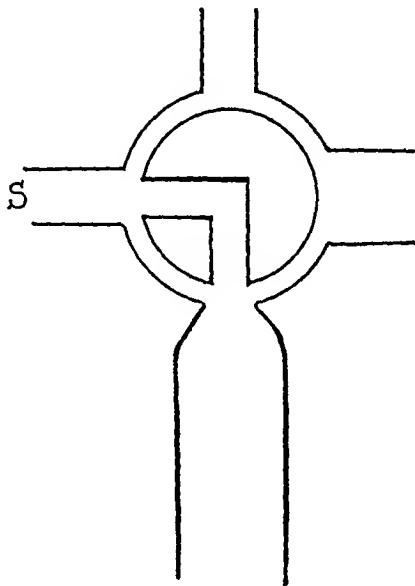


FIG. 1. Tube *S* is used to keep the pressure in the electrode equal to that of the air when the electrophoresis apparatus is not in use. For the complete description of the apparatus, the earlier publications may be consulted.

*Electrodes.*—We have continued to use  $\text{Cu-CuSO}_4$  (sat.) reversible electrodes.<sup>14</sup> Agar plugs have been discarded and in their place plaster of paris plugs have been

more useful and are more satisfactory.\* The agar plugs deteriorate quickly and must be replaced frequently. Plugs of plaster of paris, on the other hand, although having the undesirable quality of a lower electrical conductance, may be used over periods as long as 6 months without replacement if care is taken to prevent erosion by solutions employed in experimentation. The plugs are made in the fashion described previously.

*Avoidance of Turbulent Streaming.*—A further slight modification of the apparatus described previously is successful in almost completely abolishing serious

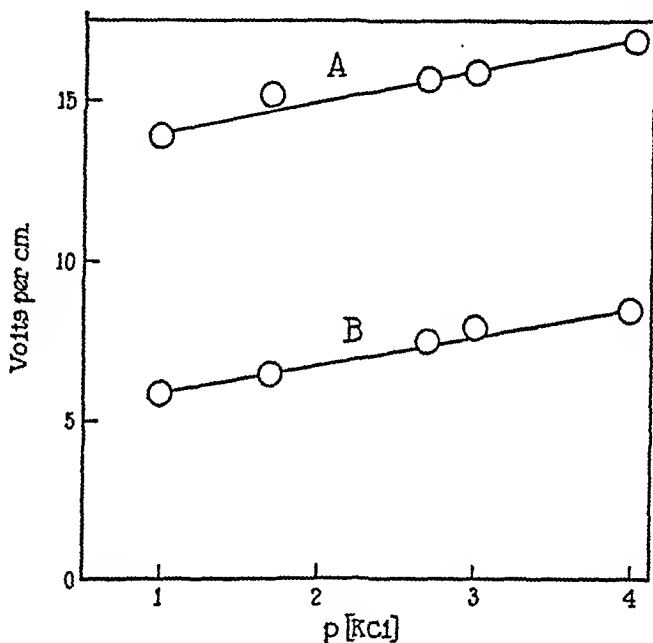


FIG. 2. The gradient of potential (with the total P.D. = 120 volts) in the cells A and B is plotted against the negative logarithm of the concentration of KCl solution filling the apparatus. The explanation for the rise in gradient of potential with dilution of salt is given in the text. See Table I.

streaming due to unequal pressures in the electrode vessels. Tube S in Fig. 1 is merely a short, small glass tube which serves to connect the electrode with the air when the apparatus is not in use. It is employed on both sides of the cell.

\* A permanent connecting plug of a porous type of Portland Cement supplied by the Aerocrete Company has been used for 1 year without solution or even erosion. This material is suitable only if very small currents need to be passed through the apparatus. If currents of the usual order of magnitude are employed polarization at the boundaries of these plugs may occur.

Since variation in room temperature causes expansion and contraction of the liquid within the electrode vessels, turbulent streaming is frequently encountered after the cell has not been in use during the night. We had previously left one electrode open through the opposite stop-cock to the outside, after filling the apparatus with saturated  $\text{CuSO}_4$  or  $\text{Na}_2\text{SO}_4$  solution. Before the apparatus could be used, however, the other electrode pressure had to be equilibrated. To dispense with the necessity for this procedure of pressure equilibration since the liquids in the electrode vessels are permanently maintained at atmospheric pressure when the apparatus is not in use.

*The Drop in Potential in the Cell.*—For the past 5 years the number of volts per cm. in the cell has been calculated by means of Ohm's law.<sup>14</sup> This method has

TABLE I

The dimensions of two microelectrophoresis cells suitable for most purposes. These cells are recommended since the differences in dimensions give a considerable difference in working range with possibility of cross-checking.

| Approximate dimensions of         | Cell A | Cell B | Remarks   |
|-----------------------------------|--------|--------|---|
|                                   | cm.    | cm.    |   |
| Bore of stop-cock.....            | 0.3    | 0.3    | It can be shown graphically that no considerable error is introduced by calculating $q$ , the cross-section, from a mean value of the thickness obtained from about ten points across the width of the cell. The cell is not exactly uniform in cross-section. Cells A and B, in addition to the differences noted, had electrode vessels of different sizes and shapes. The type recommended is indicated in Fig. 1. |
| Length of side-arm.....           | 6.5    | 8.1    |   |
| Internal diameter of side-arm.... | 0.9    | 0.6    |   |
| Length of cell.....               | 3.5    | 4.0    |   |
| Thickness of cell (mean).....     | 0.081  | 0.085  |   |
| Width of cell.....                | 0.99   | 1.15   |   |

given complete satisfaction at all times throughout this period. Cataphoresis cells varying considerably in dimensions have been used; voltages giving potential drops from 1 to 20 volts per cm. have been employed. At no time has there been any evidence that this manner of determining the potential drop within the cell is incorrect. This method is here again recommended as the most accurate and the simplest method of determining the potential gradient within the cell, since it involves only the measurement of the specific resistance of the suspension, and of the cross-section of the cell, which is made with facility on the square movable microscope stage with verniers. Fig. 2 gives an idea of the relationship between this potential gradient and the concentration of KCl solution contained in two different cells, A and B, during measurements. It is evident that the drop in potential in cell A is considerably greater than that in cell B for a given voltage. The di-

mensions of these two cells are given in Table I. Note in Fig. 2 that the drop in potential in the cell increases with the specific resistance of the liquid and within the limits of experimental error reaches a limiting value. This is easily understood on the basis of the following reasoning. The current flowing in the system is, for a given applied electromotive force, evidently determined by  $r$ , the total resistance of the apparatus,

$$r = r_1 + r_2 + r_3$$

where  $r_1$  = resistance of both electrodes

$r_2$  = resistance of both stop-cocks and of both side-arms

$r_3$  = resistance in the cell itself.

Neglecting diffusion at the stop-cock-plug junction, and assuming  $r_1 < r_2 + r_3$ , a condition found under ordinary experimental conditions, and considering that, with the increase of specific resistance of the suspension,  $\frac{r_2 + \Delta r_2}{r_3 + \Delta r_3} = \frac{r_2}{r_3}$ , we have then the conditions which account for the curves in Fig. 2, or

$$\frac{r_3 + \Delta r_3}{r_1 + (r_2 + \Delta r_2) + (r_3 + \Delta r_3)} > \frac{r_3}{r_1 + r_2 + r_3}$$

Only when  $r_1$  is so small that it can be neglected will the drop in potential within the cell be proportional to the applied electromotive force.

#### EXPERIMENTAL

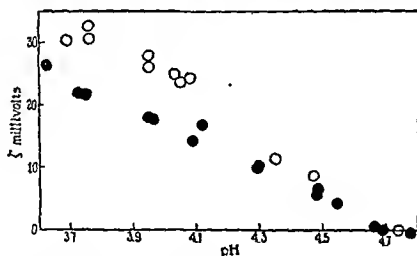
The two cataphoresis cells just described were used in the investigation of the cataphoresis of protein-covered quartz particles. The suspensions were made up as previously described and the ionic strengths and ionic species were made identical with those in Briggs' experiments. Our data are given in Table II. There is excellent agreement between Cell *A* and Cell *B* in Experiments 6, 7, and 8. Experiment 9B was the last experiment to be performed, late at night, and since it varies appreciably from the other data it is not plotted in Fig. 3. The agreement between the results with the two cells indicates that the precision obtained with the microscopic method of measuring electrical mobilities is approximately as good as that obtained by Gortner and his coworkers with the method of streaming potentials.

To compare our present data with those of Briggs<sup>2</sup> we have plotted in Fig. 3 both sets of data, calculated by means of equations (1) and (2). Our values are consistently higher than those of Briggs. The differences are about twice the extreme limits of the error inherent in

TABLE II

Electrical mobility and  $\zeta$ -potentials of protein-covered quartz particles suspended in 0.0004 M LiCl-HCl solutions. See text.

| Cell         | pH   | $\frac{V}{\mu/\text{sec}/\text{volt}/\text{cm.}}$ | volts (E.S.U. $\times 3 \times 10^3$ )     |
|--------------|------|---|--|
| 1 A          | 4.35 | -0.81   | 11.4                                       |
| 2 A          | 4.47 | -0.61   | 8.6  |
| 3 A          | 4.05 | -1.67   | 23.6                                       |
| 4 A          | 3.69 | -2.16   | 30.5                                       |
| 5 A          | 4.08 | -1.72   | 24.3                                       |
| 6 A }<br>B } | 3.95 | $\begin{cases} -1.97 \\ -1.84 \end{cases}$        | $\begin{matrix} 27.8 \\ 26.0 \end{matrix}$ |
| 7 A }<br>B } | 3.76 | $\begin{cases} -2.16 \\ -2.30 \end{cases}$        | $\begin{matrix} 30.6 \\ 32.5 \end{matrix}$ |
| 8 A }<br>B } | 4.74 | $\begin{cases} <0.1 \\ <0.1 \end{cases}$          | $\begin{matrix} <1.4 \\ <1.4 \end{matrix}$ |
| 9 A }<br>B } | 4.03 | $\begin{cases} -1.77 \\ -2.18 \end{cases}$        | $\begin{matrix} 25.0 \\ 30.8 \end{matrix}$ |

FIG. 3. ● Streaming potential (Briggs) ( $\zeta_s$ )○ Electrophoresis ( $\zeta_e$ )

The difference between  $\zeta_s$  and  $\zeta_e$  cannot be taken as real, under the experimental conditions. The data indicate rather a qualitative agreement upon which further experimentation can be planned.



the method of electrophoresis. Since it was impossible to use the same suspension of protein-covered quartz particles which Briggs employed, it is difficult to state that an actual difference as large as this exists between  $\zeta_e$  and  $\zeta_s$ , calculated as indicated. It seems likely that differences in the two samples of protein used may be responsible for the variation observed. The data at present available for protein-covered quartz surfaces have all been obtained in electrolyte solutions having too high a conductance to be used in streaming potential experiments. In the near future data suitable for comparison will be published. Meanwhile, experimentally, there is the qualitative agreement upon which more precise experimentation may be based.

#### SUMMARY

1. The conditions are described which are necessary for the comparison of certain types of electrokinetic potentials. An experimental comparison is made of (a) electrophoresis of quartz particles covered with egg albumin; and (b) similar experiments by Briggs on streaming potentials. A slight, consistent, difference is found between the electrophoretic potential and the streaming potential. This difference is probably due to the difference in the protein preparations used rather than to real difference in the electrophoretic and streaming potentials.
2. Data are given which facilitate the measurements and enhance the precision of the estimation of electrical mobilities of microscopic particles.

The authors are indebted to Professor W. J. Crozier for his careful revision of certain parts of this manuscript.

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However, in 3 weeks the indicator in the 3 per cent gel was yellow throughout, in  $2\frac{1}{2}$  months the color of the 5 per cent gel was uniform throughout but corresponded to a pH of about 4, *i.e.*, within the range of the indicator. The 8 per cent gel still had a yellow, very diffuse zone at the top.

We repeated these experiments using 0.01 N HCl whose concentration was kept constant. For this purpose a gently flowing reservoir

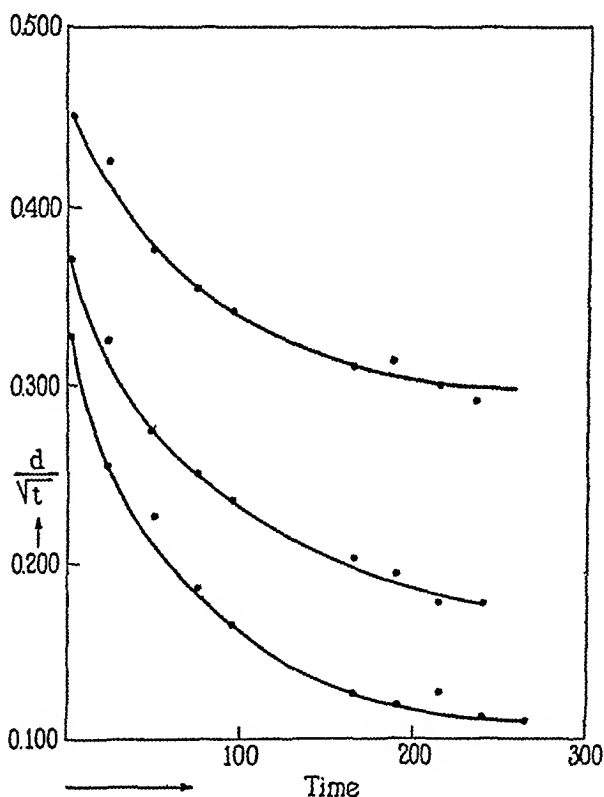


FIG. 1. .015 N HCl in limited amount

and constant level siphon system was used. It will now be observed that  $\frac{d}{\sqrt{t}}$  remains constant within experimental error (Fig. 2). Sobotka and Sabin (4) have also found that the constant gives lower values at the beginning of the experiment. If an equilibrium were being approached, one would expect  $\frac{d}{\sqrt{t}}$  to decrease continuously. The

decreasing values for the first experiments can, however, be explained as in simple diffusion by the exhaustion of the diffusing substance.

Much more precise forms of the equation  $\frac{d}{\sqrt{t}} = k$  have been formulated by Adair, Stiles, and others (5), but it was not thought necessary to use them here.

It will thus be seen that all the conditions imposed in Bigwood's experiments would lead one to expect an extreme case of slow diffusion which might be easily mistaken, with relatively crude measuring

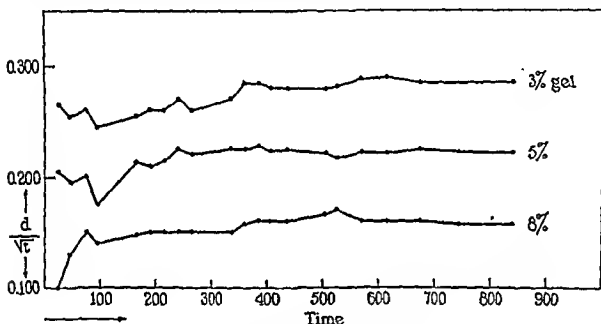


FIG. 2. 0.01 N HCl at constant concentration

methods, for a stationary system. His rather concentrated gels (8 per cent) were in contact with a limited amount of very dilute alkali (usually less than 0.004 N) whose pH, as he shows, was continuously decreasing. No mention was made of a preliminary purification of the gelatine which ordinarily contains 2-3 per cent ash. The active concentration of the alkali is further decreased by combination with the gelatine.\* Sohotka and Sabin (4) have demonstrated the influence of this factor on speed of diffusion. Using the average value for the diffusion constant found in these experiments for an 8 per cent gel

\* I cannot agree with Bigwood's conclusion that the gelatine in an 8 per cent gel is a negligible source of neutralisation for diffusing  $\text{OH}^-$  ions when the pH of the outside solution is equal to or greater than 10 (Reference 1c, p. 711).

in contact with continuously renewed 0.01 N HCl, calculations show that it would take  $3\frac{1}{2}$  months for the indicator to change throughout the 9 cm. column and that toward the end the boundary, if clear, would take 2 weeks to move 0.2 cm. It is not remarkable, then, that this should appear to be a system in stationary equilibrium.

The fact that dilute acid was used in these experiments instead of alkali should not affect the results as long as the gels were not, at the end, at the isoelectric point but within the zone of color change of the chosen indicator.

In reference to his more recent experiments Bigwood points to the shifting of ion concentration, due perhaps to the liberation of acid valency in proportion to the evolution of the gel structure. In our

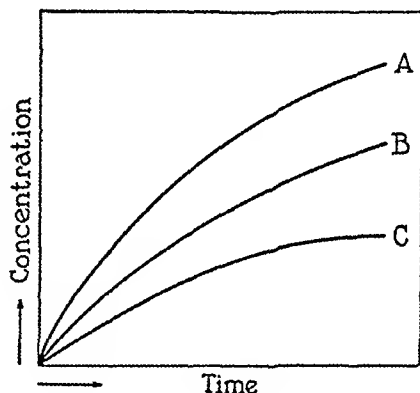


FIG. 3

opinion, this reversal of the various pH *niveaux* after a time can be explained by an examination of the ordinary form of diffusion curves for different concentrations (Fig. 3). If the concentration of diffusing substance were originally *A* and gradually dropped to *B*, the concentration at any one point in the gel would return to the value for the curve for *B* at that time. At a certain time this difference in concentration would mean a return to the original color of the indicator.

In any case, a reversal of the zone of color change after replacing the supernatant solution with water does not prove that a slow diffusion had not been taking place toward the interior of the gel. It may be a question of simply varying the relative intensity of diffusion in each direction.

The theory of the existence of a permanent concentration gradient of hydrogen ion is based on the assumption of a permanent gradient for protein micelles. Light-scattering experiments, carried on by and with the kind assistance of Dr. K. Krishnamurti in these laboratories, do not indicate such a gradient under the given conditions. When he allowed uniform gelatin gels to imbibe small amounts of distilled water and measured the light-scattering power at various heights in the column, he found that it became identical throughout if sufficient time were allowed. Similarly, he measured 5 cm. columns of 3 per cent isoelectric gelatine in contact with 8.5 cc. 0.3 N HCl. The change in turbidity was the same throughout the gel in 2 weeks. The greater concentration of acid used in this investigation allowed equilibrium to be reached in a shorter time but the method is sensitive enough to detect any appreciable gradient (6).

Since we were unconvinced that permanent concentration gradients could be obtained in this way, we prepared one by layering equal amounts of gelatin sols of varying concentrations upon each other in a test-tube, being careful to avoid air-gaps or sharp interfaces within the gel. There was a uniform concentration of brom-phenol blue in the column which was then covered with 10 cc. 0.03 N HCl. This was renewed in 4 days and removed in 11 days. After  $4\frac{1}{2}$  months a color gradient still persisted within the gel, the hydrogen ion concentration not having distributed itself evenly as had been the case with uniform gels under similar conditions. An attempt was made to test the conclusion that, with a protein concentration gradient and a diffusing electrolyte, the concentration of each ion would also be a gradient but in opposite directions. This is expressed by Donnan (2) in the following differential equations:

$$\frac{dh}{dx} = - \frac{ch}{2(1 + bh) + cp + \frac{cp}{1 + bh}} \cdot \frac{dp}{dx}$$

and

$$d(A^-) = \frac{c(A^-)}{2(1 + bh) + cp + \frac{cp}{1 + bh}} \cdot \frac{dp}{dx}$$

where  $h$  and  $A^-$  are the concentrations of the free cations and anions respectively of the added electrolyte at place  $x$  in the gel,  $c$  and  $b$  are constants in the simple Langmuir equation for the reversible adsorption of cations, and  $p$  is the micelle concentration. Therefore, if  $\frac{dp}{dx} > 0$ , then  $\frac{dh}{dx} < 0$  and  $\frac{d[A^-]}{dx} > 0$ .

After  $4\frac{1}{2}$  months the gel was removed by cracking the tube, divided into sections, and analyzed. The indicator showed a pH increasing

TABLE I

| No. | Weight of gel | Concentration<br>gelatine | Cl <sup>-</sup> (total) | Cl <sup>-</sup> per gm. gel |
|-----|---------------|---------------------------|-------------------------|-----------------------------|
|     | gm.           | gm. per cent              | mg.                     | mg.                         |
| 1   | 2.698         | 1.89                      | 1.115                   | 0.408                       |
| 2   | 3.925         | 1.87                      | 1.593                   | 0.399                       |
| 3   | 3.865         | 2.07                      | 1.752                   | 0.445                       |
| 4   | 3.497         | 2.23                      | 1.752                   | 0.493                       |
| 5   | 2.756         | 2.54                      | 1.381                   | 0.494                       |
| 6   | 4.629         | ....                      | .....                   | .....                       |
| 7   | 2.967         | 4.28                      | 2.036                   | 0.673                       |
| 8   | 4.827         | ....                      | .....                   | .....                       |
| 9   | 1.943         | 6.49                      | 1.540                   | 0.780                       |

The last column is corrected for the small blank on the gelatin. The chlorides were estimated by a method similar to that given by Van Slyke, D. D., *J. Biol. Chem.*, 1923, 58, 52.

gradually from Sections 1 to 9. Table I gives the results of analysis. Of course, a gradient of protein concentration does not necessarily mean a gradient of micelle concentration but it is unnecessary to repeat here the simplifying assumptions that were used as the basis and limitations of Donnan's theory.

It will be seen that the conclusions drawn by Donnan are verified

in a general way, since the anion ( $\text{Cl}^-$ ) concentration varies in the same direction as the protein concentration, while the cation ( $\text{H}^+$ ) varies in the opposite direction.

The author wishes to express her thanks and appreciation to Professor F. G. Donnan for the suggestion of the problem and for his constant interest and advice during the work represented by this paper.

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# THE OCCURRENCE OF PORPHYRINS IN CULTURES OF *C. DIPHThERIAE*\*

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The observation has been reported in a brief note<sup>1</sup> of a characteristic absorption spectrum in filtrates of *C. diphtheriae*. The pigment responsible for the selective absorption may be extracted from the culture liquid after this has been metabolized by the bacteria and is found to belong to the group of the porphyrins. The origin of the pigment from the cytochrome of the bacilli is therefore highly probable, if not certain. Since the constitution of cytochrome—a term which at the present time signifies only a characteristic absorption spectrum subject to oxidation-reduction change—is uncertain, it appeared desirable to undertake a more intensive investigation in the hope of throwing light on the structure and the function of this intracellular pigment. A peculiar and unexpected immunological interest arose from the relation which was observed between the presence and amount of the porphyrin and the biological reactivity of the culture filtrate.

The cultures of *C. diphtheriae* were grown according to the procedure employed for the production of diphtheria toxin. After various periods of incubation the culture liquid was passed through a Berkefeld candle. The filtrates were examined spectroscopically with a Spencer spectrometer equipped with a Wallace replica grating of 10,100 lines to the inch. Quantitative spectrum determinations were made with a large Hilger spectrometer in conjunction with a Bausch and Lomb reversible photometer. The lamp, the specimen tube support, and the various

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† A part of this work was carried out at the Physiological-Chemical Institute of the University of Hamburg, Eppendorf General Hospital (Professor Schumm), Hamburg.

<sup>1</sup> Coulter, C. B., and Stone, F. M., *Proc. Soc. Exp. Biol. and Med.*, 1930, 27, 715.

addition to that which has been referred to above and which is likewise not a porphyrin. These two pigments have similar spectrochemical properties. Further consideration of them would be without significance for the present purpose.

On the addition of  $K_3FeCN_6$  the bands at 574 and 537 completely disappear, but can be brought back by adding  $Na_2S_2O_4$ . If this experiment is repeated a number of times on the same filtrate, the bands in the reduced state fade and eventually cannot be restored. It is therefore evident that the pigment which absorbs at 574 and 537 is not autoxidizable but is capable of reversible oxidation-reduction change.

The investigation of the porphyrin pigments was carried further with their extraction from the filtrates. Our procedure was as follows: to 100 cc. of filtrate were added 10 cc. of glacial acetic acid and 30 cc. of ether in a separatory funnel, and the mixture agitated without sufficient violence to cause foaming. The purple ether layer was decanted from the layer of emulsion and precipitate, and the extraction repeated with fresh acetic acid-ether. Two or more extractions of the separated aqueous layer were required to extract the pigment completely if its amount were large. The united ether portions were washed with distilled water until nearly acid-free.

Spectroscopic examination of the ether extracts from different filtrates shows that they contain varying amounts of two porphyrin substances (Fig. 2). One of these may be separated by shaking the washed ether extract with 5 per cent HCl. This dissolves out a porphyrin in which the positions of the main bands, in comparison with the values in brackets given by Schumm<sup>2</sup> for coproporphyrin, are as follows:

In weak acetic acid-ether:

|         |         |         |         |
|---------|---------|---------|---------|
| 623.7   | 568.4   | 526.7   | 495.3   |
| (623.6) | (568.4) | (526.3) | (495. ) |

In pyridine

|         |         |         |         |
|---------|---------|---------|---------|
| 622.7   | 566.9   | 531.7   | 498.5   |
| (622.5) | (567.5) | (531.5) | (499.5) |

In M/10 NaOH

|         |         |         |         |
|---------|---------|---------|---------|
| 617.7   | 564     | 538.2   | 501.4   |
| (617.7) | (565.5) | (538.3) | (503.3) |

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<sup>2</sup> Schumm, O., *Spektrochemische Analyse*, Jena, Gustav Fischer, 2nd edition 1927.

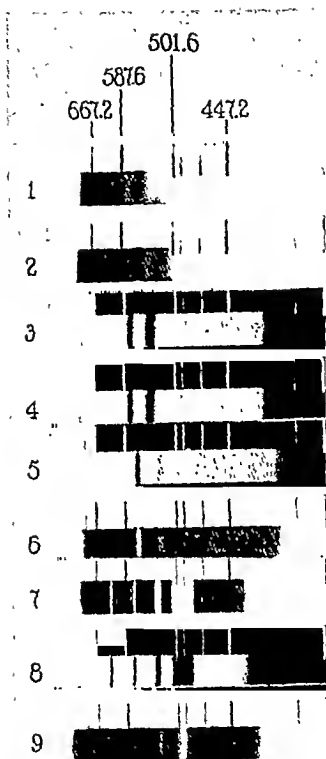


FIG. 2. Spectrograms of porphyrin

1 and 2. Porphyrin in toxic filtrate of *C. diphtheriae* (lf 6.3)

3 and 4. Complex porphyrin isolated from a filtrate.

5 and 6. Copper compound of a preparation of coproporphyrin which was obtained from a filtrate.

7, 8 and 9. Coproporphyrin isolated from a filtrate.

The porphyrins were in ethereal solution. Wratten and Wainwright panchromatic plates were used with toluidine blue filter to reduce the sensitivity of the plates to the red portion of the spectrum.

The reference lines are those of helium.

The same values within close limits are found in the solution of M/5 phosphate buffer of pH 7.5, but when dissolved in M/100 NaOH, there is a shift of the bands toward the violet, to give:

|   |         |         |     |
|---|---------|---------|-----|
| 602.2   | 461.1   | 536.8   | 501 |
| In 25 per cent HCl  |         |         |     |
| 593.4   | 549.4   | 403.    |     |
| (593.6)   | (550.2) | (405.8) |     |
| In 1/500 HCl-alcohol, treated with bromine (brom-spectral test) |         |         |     |
| 499.2   | 470.    |         |     |
| (499. )   | (470. ) |         |     |

The copper compound prepared in the usual way with the porphyrin from the filtrates, gives the following values:

|                      |         |
|----------------------|---------|
| In acetic acid-ether |         |
| 560.7                | 523.4   |
| In pyridine          |         |
| 564.8                | 528.5   |
| (563.5)              | (528.0) |

The values in brackets are those of Schumm, for the copper compound of coproporphyrin.

The HCl-soluble porphyrin of the filtrates appears therefore to be coproporphyrin.

We have not been able on account of the lack of material, to identify by means of melting-point determinations the particular isomer of coproporphyrin which is represented. We have, however, obtained the characteristic bipyramidal crystals of the hydrochloride by slow evaporation of the solution in HCl.

Quantitative determination of the extracted coproporphyrin was made on solutions in 5 per cent HCl, using the maximum of absorption at  $m\mu$  546 for spectrophotometric measurement. The results will be considered below.

The ether extract of the filtrates contains after separation of the coproporphyrin a complex porphyrin which does not correspond as far as we have been able to determine with any porphyrin which has been hitherto described. It is soluble in ether, glacial acetic acid, pyridine, and aqueous alkali; it is insoluble in 5 per cent (or stronger) HCl, but soluble in the acid form in chloroform. The solutions have a blue-violet color; the absorption bands are as follows:

|                           |       |
|---------------------------|-------|
| In weak acetic acid-ether |       |
| 574.1                     | 536.8 |

|                              |       |
|------------------------------|-------|
| In pyridine                  |       |
| 579.8                        | 541.8 |
| In $\text{M}/10$ NaOH, about |       |
| 573                          | 537   |

This porphyrin is unstable and breaks down to yield two recognizable constituents, which are coproporphyrin and the copper compound of coproporphyrin.

A solution in glacial acetic acid yields after a few hours standing a large portion of coproporphyrin and a small portion of the copper compound of coproporphyrin. These have been identified in accordance with the values given in an earlier part of this paper. The complex porphyrin appears stable, at least over a period of several weeks, in ethereal solution when washed acid-free. In acetic acid-ether solution, we have observed the gradual fading of the band at  $\text{m}\mu$  574 and the simultaneous appearance of the band at 561 of the simple copper compound. If an ethereal solution is shaken with 25 per cent HCl, the disintegration of the complex is prompt and complete. The resulting coproporphyrin passes into the aqueous phase, while the ether phase shows only the bands of the copper coproporphyrin compound. Disintegration occurs also in alkaline aqueous solution and is accelerated by light and heat. In  $\text{M}/10$  NaOH the spectrum of the unaltered complex is visible for only a few minutes before the bands of coproporphyrin appear.

In consequence of this instability, it has been so far impossible to obtain the complex porphyrin in crystalline form. The evaporated residue of an ethereal solution, although apparently stable *in vacuo*, undergoes disintegration when brought into the laboratory atmosphere.

The instability of the complex makes it difficult to detect an oxidation-reduction change. However, one can be made out if the material is kept under spectroscopic observation during all procedures and if the experiment is carried out rapidly. On the addition of a few drops of a strong solution of  $\text{K}_3\text{Fe}(\text{CN})_6$  to 3-4 cc. of a solution of the complex in  $\text{M}/10$  NaOH, the bands fade and almost completely disappear. They return to their original intensity after the addition of hydrazine hydrate. Repetition of this test leads, as in the original filtrate, to a destruction of the complex porphyrin.

The complex porphyrin is undoubtedly that constituent of the culture filtrate pigment which yields the peaks of absorption of  $\text{m}\mu$  574 and 537. This is evident from the close correspondence in the position of the bands of the extracted porphyrin with those in the filtrate at these wave-lengths. It is impossible to decide from the present data

whether or not the porphyrin exists in the filtrate in combination with some substance which is split off during extraction, although no evidence is afforded by the spectro-chemical behavior of a difference in combination between the pigment in the filtrate and in corresponding solution (with consideration of pH for example) after extraction.

Hill<sup>3</sup> has found that of the compounds of the porphyrins with the heavy metals, only those with Fe, Co, and Mn show oxidation-reduction change. This would lead us to believe that the complex porphyrin which can manifest such a change, contains Fe. Inasmuch as the porphyrin has not been obtained in crystalline form, the result of analysis is not above suspicion; the test for Fe on a digest however, was strongly positive. The complex porphyrin actively catalyzes the oxidation of phenolphthalin by  $H_2O_2$ , a result which would point further to the presence of Fe in pyrrol combination in the porphyrin, but a weak peroxidase action has been given by our preparations of coproporphyrin as well as by a crystallized preparation of copper mesoporphyrin, so that the action may be due in all of these cases to minute traces of Fe-pyrrol material present as impurity.

The origin of the copper which appears in the copper-coproporphyrin derivative of the complex is a matter of some interest. Fischer<sup>4</sup> has shown that solutions of coproporphyrin held in brass containers may form the copper compound. Throughout all of our procedures, contact of the filtrates and the extracted porphyrins with metal was avoided except during filtration when the alkaline bouillon (pH 7.2 — 8.0) came into contact with the brass stem of the filter candle. The absorption bands of the complex porphyrin are visible in the culture liquid before filtration, so that it is entirely probable that the source of the copper is the prepared culture bouillon which provides this element during the growth and autolysis of the bacteria.

The available evidence indicates that the complex porphyrin is a chemical entity, and that it contains both Fe and Cu in combination with coproporphyrin. In deliberate mixtures of the complex porphyrin and its two derivatives, and in old filtrates which have been stored for several months, the bands of coproporphyrin and its copper compound may be distinguished along with those which have been de-

<sup>3</sup> Hill, R., *Biochem. J.*, 1925, 19, 341.

<sup>4</sup> Fischer, H., and Fink, H., *Z. physiol. Chem.*, 1925, 150, 243.

scribed for the complex porphyrin. The latter cannot be regarded therefore as merely a mixture of the two derivatives, a possibility which is further excluded by its insolubility in 5 per cent HCl.

Quantitative determinations of the complex porphyrin were made on solutions in ether, using the position of the maximum absorption at 574 for spectrophotometer measurement. The results will be considered below in connection with the relation of the pigments to the biologic reactivity of the filtrates.

The identification of the extracted simple coproporphyrin with a pigment constituent of the filtrates is somewhat more difficult than in the case of the complex porphyrin. An alkaline solution of coproporphyrin as mentioned above shows a band at 617 which corresponds to a similar band in some filtrates. The bands of coproporphyrin about 561 and 537, which we have found in weakly alkaline solutions would be merged with those of the complex porphyrin, in the filtrates; a band is visible about 500, but is indistinct owing to the optical density of the filtrates in this region. The band seen at  $m\mu$  591 in the filtrates is not affected by the addition of hydrazine hydrate, but disappears on the addition of  $\text{Na}_2\text{S}_2\text{O}_4$  and is replaced by a band at 605, which may be seen in Fig. 1. It seems probable therefore that the change observed is not simply an oxidation-reduction shift. We have twice isolated hematin from filtrates which contained no other porphyrin substance, and it is possible that the band at 591 is that of a hematin which is changed by  $\text{Na}_2\text{S}_2\text{O}_4$ , with the loss of its Fe, into coproporphyrin. The band which we have seen in numerous cases, as in the series of Fig. 1, between 625 and 630 belongs apparently to coproporphyrin. Because of the inconstancy of these bands and the lack of any apparent relation to the biological reactivity of the filtrates, we have given them minor consideration.

The relation between the porphyrin in the filtrates and their biological reactivity may be analyzed graphically by plotting the content of porphyrin against the titer obtained on flocculation with antitoxic serum. This has been done in Fig. 3. The ordinates of the graph give the extinction coefficients  $\left(\log \frac{I}{I_0}\right)$  for a column 100 mm. in length of the porphyrin in solution; the abscissae give the value obtained in the flocculation titration. The solutions of the extracted porphyrins



were calculated to the original volume of the corresponding filtrates so that the optical density values refer to their concentrations in the original filtrate. The solid circles represent the extracted complex porphyrin in ethereal solution; the triangles, the extracted coproporphyrin in solution in 5 per cent HCl. The open circles represent the pigment as it exists within the filtrate; the measurements of density

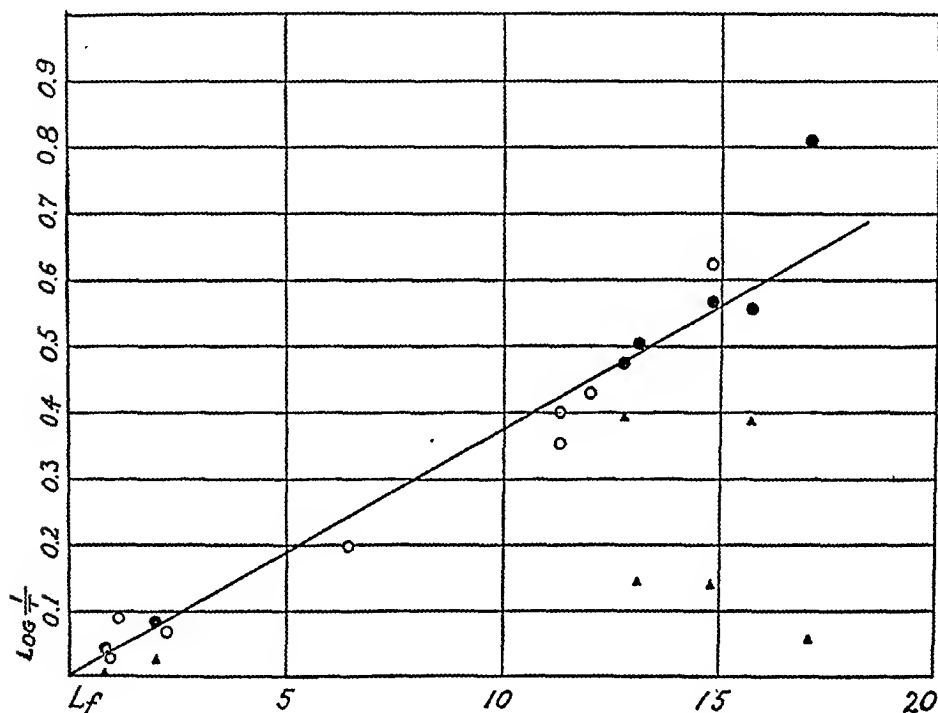


FIG. 3. Graph showing the relation between the flocculation titer and the porphyrins. Ordinates represent extinction coefficients for a column of solution, containing the pigments in the same concentration as in the original filtrate, 100 mm. in length. Abscissae represent flocculation values ( $L_f$ ). The solid circles represent extracted complex porphyrin; the open circles represent the complex porphyrin within the filtrate; the triangles represent extracted coproporphyrin.

were made at  $m\mu$  574 so that the values derive chiefly if not entirely from the complex porphyrin. The method of obtaining these values has been given above in the discussion of Fig. 1.

The values for the complex porphyrin fall along a straight line within limits which indicate a close proportionality between this porphyrin and the biologically active constituent of the filtrates. The experi-

mental error in measuring the pigment *within* the filtrate (open circles in the graph) is somewhat greater than in the case of the extracts since it is necessary to determine two values, one of which,—that for the bouillon without the porphyrin,—can only be approximated in some cases. Except for such cases, the data for all the filtrates which we have examined are included, without selection, in the graph.

The relation between porphyrin and biologic reactivity appears more striking when it is understood that the filtrates which provide the data for Fig. 3, were prepared under a wide variety of conditions. Eleven lots of bouillon with five different peptones, and four strains of *C. diphtheriae* are represented. The incubation periods varied from 3 to 13 days.

No relation is evident between the extracted coproporphyrin (triangles in the graph) and the biologically active constituent. The values for this pigment are distributed almost at random, although in general with higher flocculation values there is found more of this porphyrin.

The correspondence which we have found between the flocculation titer and the content of complex porphyrin under varied conditions has the degree of closeness indicated in Fig. 2, only when the cultures have been grown in 250 to 300 cc. amounts. We have found in a few experiments that if the volume of the original culture be 600 cc., the content of complex porphyrin is smaller relative to the flocculation titer than in cultures grown in smaller volume. The ratio of total porphyrin to this titer is the same as in the smaller volumes, in which the proportionality of total porphyrin to flocculation titer is not as close as in the case of the complex porphyrin alone. This suggests that a relation similar to that between toxin and toxoid is involved in the distribution of pigment between simple and complex porphyrin.

The porphyrin is carried down along with the toxin in precipitation by acid. The porphyrin also follows the toxin in the method of Gross,<sup>5</sup> which employs adsorption by  $Mg(OH)_2$  for the purification of toxin. Antitoxic serum however in optimal amount causes flocculation of the biologically active constituents of the toxic filtrate but leaves the porphyrin in the supernatant fluid. The porphyrin is therefore not combined with the toxin as this exists in the filtrate.

<sup>5</sup> Gross, P., *Proc. Soc. Exp. Biol. and Med.*, 1928-29, 26, 696.

Locke and Main<sup>6</sup> have found that toxin concentrated by acid precipitation shows a ratio of copper to iron of 40:1 compared to 10:1 in the uninoculated medium. This accumulation of copper in the toxin precipitate may be explained by the association of the toxin with the complex porphyrin which contains copper. Since the freshly grown bacilli according to Locke and Main show no accumulation of copper, it is possible that the porphyrin combines with the copper of the culture medium only after the liberation of the pigment from the bacilli. In view of the fact, however, that the complex porphyrin which contains copper shows an oxidation-reduction change and yields iron on analysis, it seems probable that the combination with copper takes place when the coproporphyrin is still combined with iron in the form of hemochromogen or natural porphyratin (Schumm<sup>7</sup>).

#### DISCUSSION

Coproporphyrin was recognized as a product of bacterial metabolism by Fischer and Schneller,<sup>8</sup> who obtained it from yeast. It occurs also in old tuberculin (Fischer and Fink<sup>4</sup>). The occurrence in feces and in materials which have been exposed to putrefaction suggests that it has in these cases also a bacterial derivation. The amount of this porphyrin which is produced in favorable cultures of *C. diphtheriae* is very large and far in excess of that which we have been able to extract from much larger amounts of baker's yeast.

Coproporphyrin may therefore be regarded as a component, perhaps the fundamental pyrrol constituent, of cytochrome. Its liberation in cultures of *C. diphtheriae*, in contrast to the drastic chemical procedures by which it may be produced from other sources, takes place as a result of natural processes. It is entirely probable that these involve an enzymatic cleavage of the substance of the bacteria. We have not been able to derive more than traces of porphyrin by any chemical extraction from the masses of freshly grown diphtheria bacilli.

Porphyrin has been extracted from diphtheria filtrates by Campbell-Smith,<sup>9</sup> who did not, however, find any relation between the pigment

<sup>6</sup> Locke, A., and Main, E. R., *J. Infect. Dis.*, 1930, 46, 393.

<sup>7</sup> Schumm, O., *Z. physiol. Chem.*, 1926, 152, 147.

<sup>8</sup> Fischer, H., and Schneller, K., *Z. physiol. Chem.*, 1924, 135, 253.

<sup>9</sup> Smith, F. Campbell-, *Lancet*, March 8, 1930, 1, 529.

and the biological titer. The spectroscopic values for the ether extract which Campbell-Smith has kindly communicated to us, are as follows: 623, 573-570, 537-525, together with the common porphyrin band at 410. It is entirely probable from consideration of this spectrum, that these extracts contained the porphyrins which we have described here.

The porphyrin as it occurs in our filtrates is not for the most part in its simplest form, but appears to be combined with iron, and contains copper. The degradation of the complex porphyrin to coproporphyrin takes place with such readiness that it seems extraordinary that the cleavage of cytochrome should proceed only this far without being carried promptly to the final stage of coproporphyrin.

The copper content of the complex porphyrin is a matter of interest in view of the analytical association of copper with hemoglobin and the effect of copper on the regeneration of hemoglobin (Elvehjem<sup>10</sup>). Although the purest samples of hemoglobin contain the least copper on analysis,<sup>10</sup> it is possible that a copper-containing porphyrin is the nucleus in the formation of the more complex respiratory pigments. In the case of the porphyrin it is not apparently a case of salt formation with the copper cation, since we have not been able to obtain the test for copper ion.

Although it appears certain, chiefly from the survival of the porphyrin under conditions which attenuate or destroy the toxin, that the complex porphyrin is not itself the toxic principle, the relation between the two makes it seem probable that the toxin is elaborated by the same process which yields the porphyrin. The toxin may conceivably represent the nitrogenous base,<sup>11</sup> or a fraction of it, which is combined with the porphyrin in the complex structure of cytochrome.

#### SUMMARY

A complex porphyrin which has not been hitherto described is found in liquid cultures of *C. diphtheriae*. The porphyrin is a combination of coproporphyrin with both iron and copper, and shows an oxidation-reduction change. This is the first report, as far as we are able to determine, of a porphyrin compound which contains both iron and

<sup>10</sup> Elvehjem, C. A., *J. Biol. Chem.*, 1931, 90, 111.

<sup>11</sup> Anson, M. L., and Mirsky, A. E., *J. Gen. Physiol.*, 1928-29, 12, 273

copper in its molecule. Its source is in all probability the cytochrome of the bacilli.

The content of porphyrin is proportional to the biological titer of the culture filtrate; the suggestion is offered that the toxin itself is derived from cytochrome.

We wish to thank Prof. Harold W. Webb, of the Department of Physics for invaluable assistance and advice, and to thank Dr. Elizabeth Lee Hazen of this Department, for supplying us with a number of filtrates.

# PROTEIN COAGULATION AND ITS REVERSAL

## THE IDENTITY OF NORMAL HEMOGLOBIN WITH THE HEMOGLOBIN PREPARED BY THE REVERSAL OF COAGULATION, AS DETERMINED BY SOLUBILITY TESTS

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### INTRODUCTION

Experiments which lead to the conclusion that the coagulation of proteins is reversible have been presented in previous papers (review and literature in Anson and Mirsky, 1931). From coagulated hemoglobin, globin, and serum albumin one can again prepare soluble, crystalline protein. In order to prove that coagulation is reversible it is necessary to show that the soluble protein finally obtained from completely coagulated protein is identical with the original native protein. Actually, within the limits of error, "reversed" hemoglobin has the same temperature of coagulation, the same spectrum, and the same gas affinities as normal hemoglobin and, although no exact measurements have been made, it appears to have the same crystalline form (Mirsky and Anson, 1929). The structural changes which take place during denaturation and its reversal are not completely known. It is accordingly desirable to have some means of detecting any possible difference of unknown nature between normal and "reversed" hemoglobin. Since the solubility of a protein depends on all of its groups, if "reversed" and native hemoglobin were different in any way, then theoretically their solubilities ought to be different. The experiments to be described show that within less than two per cent their solubilities are the same. Furthermore, native globin has been prepared from coagulated globin and the hemoglobin synthesized from this "reversed" globin and heme has the same solubility as normal hemoglobin.

There is no normal globin with which "reversed" globin could be directly compared because globin is denatured in the process of its preparation from hemoglobin. Finally, the solubility of a mixture of solid normal, "reversed" and synthetic hemoglobins is the same as the solubility of any one of these proteins alone.

### *Method*

In general, in making a solubility measurement, it is necessary first to prepare a suspension of the solid in a solvent of definite composition and, secondly, to saturate the solvent with the solid. With an unstable protein such as methemoglobin,<sup>1</sup> unless the procedures are carried out quickly and gently the protein changes in solubility during the experiment itself. To avoid such changes as much as possible the present measurements have been made with amorphous methemoglobin which is formed and dissolved much more readily than crystalline methemoglobin. The use of the amorphous form of methemoglobin, however, introduces a new technical difficulty. It is a familiar fact, although we have not found any quantitative study of it, that any solid is more soluble when amorphous than when crystalline. In the case of methemoglobin the difference is extreme. A saturated solution of amorphous methemoglobin is some fifty times supersaturated in respect to crystalline methemoglobin.<sup>2</sup> With time, therefore, methemoglobin crystallizes out of a saturated solution of the amorphous protein. The measurement of the solubility of amorphous methemoglobin must be finished before this crystallization has taken place to any significant extent.

<sup>1</sup> Methemoglobin has been used rather than the more stable carbon monoxide hemoglobin because it is methemoglobin which is obtained on the reversal of coagulation. The preparation of pure carbon monoxide hemoglobin from methemoglobin presents greater experimental difficulties than does the instability of methemoglobin.

<sup>2</sup> Methemoglobin has about the same solubility as oxyhemoglobin. Oxyhemoglobin has been supposed to be less soluble only because the solubility of the crystalline form of oxyhemoglobin has been compared with that of the amorphous form of methemoglobin. Under suitable conditions methemoglobin can be crystallized with ease in any amount from dilute or concentrated, isoelectric or not isoelectric solutions.

*Preparation of Suspension.*—The preparation of the suspension of amorphous methemoglobin in a solvent of definite composition is a simple matter. First the hemoglobin is brought to a definite hydrogen ion concentration by the addition of a phosphate buffer. Conditions are made as favorable as possible to secure this result. The protein is first freed of salts by dialysis. The buffer used has almost the same hydrogen ion concentration as the dialyzed protein, which fortunately is also the hydrogen ion concentration at which the buffer has its maximum buffering efficiency. And the concentration of the buffer is relatively high while that of the protein is relatively low. After the protein has been brought to a definite hydrogen ion concentration, it is salted out by the addition of concentrated ammonium sulfate. If the solutions are first cooled, it is possible to mix them completely before any precipitation whatsoever takes place. When the temperature is raised to 25°C. almost all the protein precipitates in the form of very fine particles which do not settle. The solvent held by the precipitate is the same as that in the bulk of the solution. This condition, in most solubility experiments, is achieved only by long and repeated washings; a procedure unsuitable for unstable proteins and out of the question if the crystallization of methemoglobin is to be avoided.

*Equilibration.*—The suspension which has just been described is formed by the precipitation of hemoglobin from a supersaturated solution. In the presence of a large excess of so fine a precipitate the solution cannot remain supersaturated. After only a few minutes gentle shaking the solution is in equilibrium with the solid. To make sure that equilibrium has been reached, the equilibrium is in every case also approached from below by shaking the suspension with an equal volume of solvent. The hemoglobin content of the filtrate is the same whether the equilibrium is approached from above or below.

*Avoidance of Crystallization.*—Finally, we shall state the precautions taken to avoid crystallization of methemoglobin from the saturated solution of amorphous methemoglobin and the evidence that in fact such crystallization does not take place. The salt concentration of the solvent is so chosen that the solubility of the protein is only 0.4 mg. per ml. From so dilute a solution crystallization is slow. In time, nevertheless, crystallization does take place from even a dilute solution. The filtration, therefore, must not take too long. This means that



the suspension of precipitate must not be too concentrated, for the more concentrated the suspension, the slower the filtration. With the less than 1 per cent suspension used filtration is sufficiently fast. It is possible to have a dilute suspension and still have the large excess of solid needed for quick equilibration because the solubility is made extremely low.

That no significant amount of crystallization takes place under the conditions chosen to make crystallization relatively slow and filtration relatively fast is shown by the facts that successive portions of filtrate have the same methemoglobin concentration and that the same solubility value is obtained whether the equilibrium is approached from above or from below. When visible crystallization does begin, successive portions of filtrate contain less and less protein, and less and less protein dissolves when the suspension is shaken with solvent. It may be that small, non-detectable amounts of hemoglobin crystals are formed even during the experiment but that amorphous hemoglobin dissolves to maintain the equilibrium just as fast as the solution loses hemoglobin by crystallization.

## RESULTS

As may be seen from the table, the solubilities of normal, "reversed," and synthetic hemoglobins and of the mixture of all three solids are the same within 2 per cent. In general, even small differences between two proteins are reflected in differences in solubility much greater than 2 per cent. The solubilities of horse and ox hemoglobins are so different they cannot be measured under the same conditions. It is not excluded, nevertheless, that normal and "reversed" hemoglobins are slightly different and have slight differences in solubility which fall within the experimental error. The problem of the solubilities of slightly different proteins, such as horse and mule hemoglobins, or the different derivatives of horse hemoglobin, will be discussed experimentally in a later paper. What one can conclude from the present result is that if normal and "reversed" hemoglobins are not completely identical, they are, at least, nearly the same. With the methods now available it is not possible to prove beyond doubt that any two proteins are completely identical. All one can show is that they cannot be distinguished by certain specific tests.

It remains to discuss the significance or rather the lack of significance of the measurement of the mixed solubility of normal, "reversed," and synthetic hemoglobins. According to the phase rule the solubilities of different solids suspended together in the same solvent are independent; the total amount of substance dissolved is equal to the sum of the individual solubilities of the solids. This is true regardless of how small the differences are between the solids provided only that the substances are present as individual solid phases. The fact, then, that the solubilities of normal, "reversed," and synthetic hemoglobins are not additive might be considered as proof that these three hemoglobins do not differ even slightly. Actually, however, as will be seen later, slightly different amorphous hemoglobins do act on one another to form solid solutions.<sup>3</sup> In a suspension of three slightly different hemoglobins there is only one solid phase, the solid solution of the three hemoglobins. The solubility observed is that of this one phase,

TABLE

|  | Normal | Reversed | Synthetic | Mixed |
|--|--------|----------|-----------|-------|
| Solubility, mg. Hb per ml. filtrate..... | 4.09   | 4.08     | 4.05      | 4.00  |

not the sum of the individual solubilities of the three hemoglobins. The experiment on the solubility of the mixture of normal, "reversed," and synthetic hemoglobins does *not* provide, therefore, any additional evidence for their identity.

#### EXPERIMENTAL

*Preparation of Hemoglobin.*—Distilled water is added to washed horse corpuscles to make the hemoglobin concentration about 12 per cent. The solution is shaken vigorously with a fifth its volume of toluol and left overnight in the cold. The upper layer of toluol and stromata is then removed, and the rest shaken with a seventh of its volume of centrifuged alumina cream (Tracy and Welker, 1915), allowed to stand an hour or more and filtered. Finally to avoid crystallization the hemoglobin is diluted to 10 per cent and stored in the cold under toluol.

If carbon monoxide hemoglobin is desired the blood is immediately saturated

<sup>3</sup> Evidence suggesting that the crystalline forms of slightly different hemoglobins form solid solutions has already been given by Landsteiner and Heidelberger, (1923).

with carbon monoxide and the solution is always kept under carbon monoxide. There are slight differences in the preparation of horse and ox hemoglobins. Horse corpuscles settle from serum of their own accord in a few hours while ox corpuscles have to be centrifuged. Horse corpuscles can be separated from sodium chloride solution with a Sharples centrifuge without any large proportion being broken, while ox corpuscles are broken by this procedure. A twentieth of the total volume of alumina cream suffices to secure good filtration of ox hemoglobin while much more is needed to secure complete and immediate separation of the toluol globules from horse hemoglobin solutions.

*Nitrogen Estimation.*—The Kjeldahl determinations are done with mercuric oxide as catalyst. As Dr. Northrop has observed, complete digestion of hemoglobin is not obtained with copper. Hemoglobin foams much more than do other common proteins. With small Kjeldahl flasks it is impossible to obtain accurate results unless this foaming is avoided. The Kjeldahl flask containing the solution to be estimated plus a drop of sulfuric acid may be left in an oven until practically all the water is evaporated;<sup>4</sup> or all the sulfuric acid may be added to the hemoglobin and the acid solution left in the oven until the hemoglobin has been hydrolyzed past the foaming stage. It is better to use the smaller amount of acid if the flask is to be left in the oven a short time, and the larger amount of acid if the flask is to be left in the oven a long time.

*Preparation of Normal Methemoglobin.*—To each 100 ml. of 10 per cent carbon monoxide hemoglobin is added at room temperature 0.7 gm. powdered potassium ferricyanide. The resulting methemoglobin is dialyzed overnight in the cold in a shaking dialyzer (Kunitz and Simms, 1928) against running distilled water. If a 15 per cent solution of methemoglobin is dialyzed a good deal of the protein crystallizes out.

*Preparation of "Reversed" Methemoglobin.*—The procedure is practically the same as that already described (Mirsky and Anson, 1930). To a 10 per cent solution of carbon monoxide hemoglobin at 5°C. is added three times its volume of 1/15 N HCl.<sup>5</sup> 3 minutes later 15 ml. of 0.1 N NaOH is added slowly with stirring to each 40 ml. of acid hemoglobin solution. After the partially neutralized solution has stood half an hour or more at room temperature the neutralization is completed by the addition of 5 ml. of 0.1 N NaOH for every 15 ml. already added.

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<sup>4</sup> In the test tube digestion of proteins with a mixture of sulfuric and phosphoric acids preliminary to direct Nesslerization, serious foaming occurs with all the proteins we have tried. Evaporation before digestion simplifies the procedure greatly especially if a large number of determinations are being made. If time is not important slow, partial digestion at 100°C. is also desirable before the final completion of digestion at boiling temperature. At 100°C. the phosphoric acid does not attack Pyrex glass.

<sup>5</sup> Through error, it was stated on page 478, line 10 of a previous paper (Mirsky and Anson, 1930) that an equal volume of acid was added. In other parts of that paper the solution was described correctly.

The solution is half saturated with ammonium sulfate and the precipitated denatured protein filtered off. To each 100 ml. of filtrate is added 16 gm. of solid ammonium sulfate. The resulting amorphous precipitate is filtered off and dialyzed against distilled water. The "reversed" methemoglobin may be crystallized either by adding 7.5 gm. instead of 16 gm. of ammonium sulfate to each 100 ml. of the solution half saturated with ammonium sulfate or by making a concentrated saturated solution of the amorphous hemoglobin.<sup>6</sup>

*Preparation of Synthetic Methemoglobin.*—To each 100 ml. of a 5 per cent solution of the acid acetone powder of denatured globin (Anson and Mirsky, 1930) is added first 48 ml. of 0.1 N NaOH and after 20 minutes 16.5 ml. more. The precipitate of denatured globin is filtered off. To each 100 ml. of the filtrate, which contains the native globin prepared by the reversal of coagulation and the denatured globin not precipitated by neutralization, is added 0.106 gm. of recrystallized hemin (Eastman) dissolved in three equivalents of NaOH. This amount of hemin could convert into hemoglobin all the globin contained in the original preparation. Half saturation of the solution with ammonium sulfate now precipitates any remaining denatured globin and any free heme not combined with globin. The rest of the procedure is the same as with "reversed" methemoglobin. Synthetic like "reversed" methemoglobin may be completely crystallized.

*The Phosphate and Ammonium Sulfate Solutions.*—The stock 3.6 M phosphate solution contains per liter 313.7 gm.  $K_2HPO_4$  and 245.1 gm.  $KH_2PO_4$ . To get a 1.25 M  $PO_4$  solution, 132.4 gm. of water are added to 100.0 gm. of 3.6 M  $PO_4$ . The 2 per cent methemoglobin solution contains 2 gm. (1.5 ml.) of hemoglobin to 113.5 gm. (100.0 ml.) of 1.25 M  $PO_4$ . The ammonium sulfate solution contains 533 gm. per liter. This about corresponds to a saturated solution at 15°C., a temperature which the solution may reach during the night. Since the same salt solutions were used in any given group of solubility measurements and only comparative values were desired, no check was made on the purity or dryness of the salts.

To precipitate the methemoglobin 10 gm. ammonium sulfate solution are added to each 6 gm. of 2 per cent hemoglobin in 1.25 M  $PO_4$ . The solvent then contains 10 gm. ammonium sulfate solution to each 5.90 gm. of 1.25 M  $PO_4$ .

All weighings are made easily and rapidly on a Curie balance to one part in five hundred. There is no significant loss or gain of water during the weighings. The solutions for the solubility experiment itself are weighed out in 50 ml. Pyrex Erlenmeyer flasks.

*Mixing, Shaking, and Filling.*—The solutions of 2 per cent hemoglobin and ammonium sulfate are cooled in ice water. If the ammonium sulfate solution is left cold too long the salt will of course crystallize out. The flasks are removed from the ice water, dried, and their edges covered with thin films of vaseline.

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<sup>6</sup> This is a sure and general way of crystallizing any protein whose amorphous and crystalline forms have very different solubilities. It works very well with serum albumin.

The solutions are mixed by being poured from one flask to the other and the mixed solution is brought to 25°C. and rocked gently for 8 minutes. Whether shorter times would suffice to secure equilibrium was not tried. Finally the solution is filtered through a folded No. 42 Whatman paper 7 cm. in diameter, the funnel being covered with a small Petri dish. The first 3 ml. portion is rejected because of adsorption of the hemoglobin by the filter paper. The next two portions are collected for the estimation of hemoglobin.

*Estimation.*—The hemoglobin is estimated colorimetrically as cyan methemoglobin. A blue glass is placed in the eyepiece of the colofimeter to make the estimation of the red pigment easier and more sensitive. The methemoglobin is converted into cyan methemoglobin by the addition of a trace of solid KCN.

#### CONCLUSIONS

1. Methemoglobin prepared from coagulated hemoglobin by the reversal of coagulation has the same solubility within 2 per cent as normal methemoglobin.
2. Methemoglobin synthesized from hemin and the native globin prepared by the reversal of coagulation of globin likewise has the same solubility as normal methemoglobin.

We are indebted to Dr. John H. Northrop for his helpful suggestions.

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# PROTEIN COAGULATION AND ITS REVERSAL

## GLOBIN

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It has already been shown that the coagulation of hemoglobin is reversible (review and literature in Anson and Mirsky, 1931). Hemoglobin, however, is a conjugated protein consisting of the simple colorless protein, globin, combined with the red iron porphyrin complex, heme. It might be supposed that the result obtained from the study of the reversibility of the coagulation of hemoglobin reflects not a property of proteins in general but a peculiarity which hemoglobin displays because it contains heme. We have accordingly studied the simple protein, globin, itself. If acid acetone under certain conditions is added to hemoglobin, the heme and the globin are separated, the heme remaining in solution and the globin being precipitated. By a suitable neutralization procedure it is possible to obtain from this acid acetone globin, with a yield of about 65 per cent, soluble, heat coagulable globin which can combine with heme to form crystallizable hemoglobin (Anson and Mirsky, 1930). The facts that acid acetone is in general a denaturing agent and that the denaturation of hemoglobin can be reversed by exactly the same kind of neutralization procedure used to prepare soluble globin (Mirsky and Anson, 1930) suggest that the preparation of soluble globin from acid acetone globin consists essentially in the reversal of denaturation. In confirmation of this view, the present experiments show that acid acetone globin is denatured globin and that soluble globin can be prepared from globin which has been precipitated with trichloroacetic acid or heated. The opinion of Hill and Holden (1927) that the preparation of soluble globin depends solely on the avoidance of denaturation is shown not to be justified by their experiments.

*Evidence That Acid Acetone Globin is Denatured Globin.*—Native proteins in general are soluble at their isoelectric points. Denatured proteins are insoluble at their isoelectric points but soluble in acid or alkali. The solubility of acid acetone globin is that of a denatured protein. As prepared from hemoglobin acid acetone globin is a compound of globin with hydrochloric acid which dissolves in water to give a strongly acid solution. It is completely precipitated by rapid, complete neutralization of the acid with a buffer salt and almost completely precipitated by rapid neutralization with NaOH. For instance, if an equal volume of 1 M  $K_2HPO_4$  is added to a 5 per cent solution of acid acetone globin, the filtrate gives only a slight haze with trichloroacetic acid. It is *a priori* possible that denatured proteins when precipitated carry down any native, soluble protein present and that therefore complete precipitation at the isoelectric point is not proof of complete insolubility and denaturation. Experimentally, however, we have not been able to obtain evidence that any significant amount of carrying down takes place under the conditions of our experiments. When denatured hemoglobin is precipitated in the presence of native hemoglobin, the concentration of native hemoglobin in solution is not changed (Anson and Mirsky, 1929). Similarly, if one adds ammonium sulfate at 0°C. to a mixture of heat-denatured globin (heated in a solution acid enough to prevent precipitation) and unheated globin in acid, all the heated globin is precipitated and all the unheated globin remains in solution. Northrop (1930) has done essentially the same experiment and obtained the same result with known mixtures of native and denatured pepsin globulin, and he has shown further that when a given fraction of pepsin is denatured by alkali the fraction denatured as estimated by loss of enzyme activity without any precipitation whatsoever, is the same as the fraction denatured as estimated by the amount of protein nitrogen remaining in solution after the denatured pepsin has been precipitated at the isoelectric point. These experiments do not mean that coagulated protein cannot adsorb native protein at all. Such adsorption does take place. But it is significant in amount only when the concentration of coagulated protein is very great in comparison with that of the native protein.

In general when proteins are denatured there becomes free a number of SH and S-S groups which is equivalent to the total number of cysteine

and cystine groups in the protein.\* The acid acetone globin has the number of free SH and S-S groups characteristic of denatured proteins (unpublished experiments). Meldrum and Dixon (1930) state that denatured globin has no sulphydryl groups. A simple qualitative test with nitroprusside shows that such groups are in fact present.

Finally, if the native globin obtained from acid acetone globin were simply native globin which had escaped denaturation, then the further treatment of the globin with trichloroacetic acid or heat, both well known denaturing agents, ought to result in further denaturation and hence in a much lower yield of soluble globin. In fact, as will now be seen, trichloroacetic acid and heat have little effect on the yield.

*Reversal Experiments with Globin Precipitated by Trichloroacetic Acid.*—If the acid acetone globin is rapidly completely neutralized, as has just been seen, it is almost completely precipitated. If it is first only partially neutralized, a little less than enough alkali to cause incipient precipitation being added, then about two-thirds of the globin is found to be soluble native globin when after a time the neutralization is completed (Anson and Mirsky, 1930). If enough alkali is first added to precipitate and just redissolve the globin, then about one-third of the globin is found to be soluble in 0.4 saturated ammonium sulfate. Reversal on the alkaline side of the isoelectric point with globin gives a lower yield than reversal on the acid side. Since trichloroacetic acid denatures proteins in acid solution, in the presence of trichloroacetic acid only the alkaline reversal procedure is possible. When this is tried with a trichloroacetic precipitate the same 33 per cent yield is obtained as from the globin to which no trichloroacetic acid has been added. The quantities used are as follows. To 15 ml. of a 3.3 per cent of acid acetone horse globin is added 25 ml. of 2.2 per cent trichloroacetic acid. The centrifuged precipitate is suspended in 10 ml. water and dissolved with 13 ml. of 0.1 N NaOH. After a few minutes 0.1 N HCl is added to give a heavy precipitate and the solution is 0.4 saturated with ammonium sulfate. The yield is estimated by the procedure already described (Anson and Mirsky, 1930).

Experiments exactly the same as the one just described can be done with hemoglobin (Mirsky and Anson, 1930) and serum albumin (unpublished experiments).

\* See Mirsky, A. E., and Anson, M. L., 1930, *Proc. Soc. Exp. Biol. and Med.*, 28, 170.



*Reversal Experiments with Heated Globin.*—A 5 per cent acid solution of the acid acetone ox globin is heated in boiling water for 3 minutes. By the neutralization procedure a 60 per cent yield of soluble globin is obtained. Again the same experiment can be done with hemoglobin and serum albumin.

Soluble globin can likewise be obtained from acid acetone globin prepared from hemoglobin heat coagulated in neutral solution.

*Hill and Holden's Theory.*—After it was shown (Anson and Mirsky, 1925) that globin prepared by the classical method of Schulz (1898) is denatured globin and that hemoglobin is a compound of native globin and heme while the hemochromogen prepared from hemoglobin is a compound of denatured globin and heme, Hill and Holden (1927) devised a method for preparing native globin. They assumed to begin with that coagulation cannot be reversed and that therefore the mere fact that they obtained native globin was in itself *a priori* proof that their native globin had never been denatured. They did not carry out any experimental tests to see whether the globin in fact had escaped denaturation. Their procedure involved extreme precautions to avoid denaturation, such as the careful maintenance of low temperature during the separation of globin from hemoglobin, the use of an atmosphere free from organic materials, etc. These precautions supposedly were the essential elements of the procedure. Actually these precautions are entirely unnecessary. We have found that by dialyzing globin against cold distilled water one can get the same 60 per cent yield of soluble protein whether one carries out Hill and Holden's procedure for separating heme and globin in the cold or at room temperature. The 60 per cent yield obtained at room temperature is higher than the one Hill and Holden reported for their preparation in the cold. And the yield they reported was higher than the one they obtained since in estimating how much denatured protein remained they did not precipitate the denatured protein completely. The opinion that the denaturation which admittedly takes place in acid at room temperature prevents the obtaining of soluble globin on neutralization is therefore not correct. The differences in yields in the different preparations are due to different conditions of neutralization, not to the greater or less avoidance of denaturation. Since there is no evidence in Hill and Holden's experiments that they avoided dena-

turation and since the present experiments show that one can obtain soluble globin from globin which has been denatured, it is not decided by Hill and Holden's experiments whether or not they were correct in their theory that native globin can be separated directly from hemoglobin. An observation of Holden and Freeman (1928) suggests, however, that Hill and Holden did denature their globin. If Hill and Holden's acid solution of globin is 1/100 saturated with ammonium sulfate then no soluble globin is obtained on neutralization. To explain this fact, Holden and Freeman had to assume that, contrary to experience, this small amount of salt actually brings about a rapid denaturation of the protein. A simpler explanation and one in accord with experience, is that the salt precipitates the already denatured protein, and thereby prevents reversal of denaturation.

#### CONCLUSIONS

1. The globin prepared from hemoglobin by the acid acetone method is denatured globin.
2. The denaturation and coagulation of globin by acid acetone are reversible.
3. Soluble globin can be obtained from the acid acetone globin even if the globin is first precipitated by trichloroacetic acid or heated to 100°C.
4. Hill and Holden's theory that they separated native globin from hemoglobin without any intermediate denaturation is not proven by their experiments.

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# THE PRODUCTION AND INHIBITION OF ACTION CURRENTS BY ALCOHOL

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This paper describes an investigation growing out of preliminary experiments begun in 1922 by Mr. E. S. Harris and the senior author.<sup>1</sup> It deals with lowering of P.D. by virtue of which alcohol is able to start and stop action currents in *Nitella*.

It has already been stated that chloroform and KCl can stimulate by lowering the P.D. at a given spot until the inflow of current from neighboring regions is sufficient to start a negative variation.

Ethyl alcohol can act in the same manner. An interesting example of this appears in Fig. 1.<sup>2</sup> The alcohol was applied near the center of the cell, as shown in Fig. 2, at the spot marked *B*. At the start the P.D. of *B* with reference to *D*, as shown by curve *B*, was about 100 millivolts positive, owing to the fact that *D* had previously been killed by chloroform.<sup>1,3</sup> When alcohol (1.5 M) was applied the P.D. gradually fell toward zero (as shown by the rise of the curve) and when it reached 80 millivolts an action current started as shown by the sudden jump of the curve. The excitation spread in both directions, as shown by the movements of curves *A* and *C* about a second later. At *A* and *C* recovery was normal but at *B* it was very slow. At the point marked 2 on the record the cell was stimulated electrically at *X*. A normal

<sup>1</sup>Osterhout, W. J. V., and Harris, E. S., *J. Gen. Physiol.*, 1927-28, 11, 673.

<sup>2</sup>This record was chosen for reproduction from many which were similar. The statements made about it apply qualitatively to all the others, and are in good agreement quantitatively.

<sup>3</sup>The experiments were performed on *Nitella flexilis* with the technique previously described (Osterhout, W. J. V., and Harris, E. S., *J. Gen. Physiol.*, 1928-29, 12, 167, 355; Osterhout, W. J. V., and Hill, S. E., *J. Gen. Physiol.*, 1930-31, 14, 385). The method of measurement is essentially electrostatic. The temperature was very close to 20°C.

response is seen at *A* but only a very slight one at *B*: since the impulse does not pass on to *C* it is evident that the alcohol at *B* is acting as a block.

At the point marked 3 the shutter of the camera was closed and the motion of the photographic paper was stopped for 1 minute during

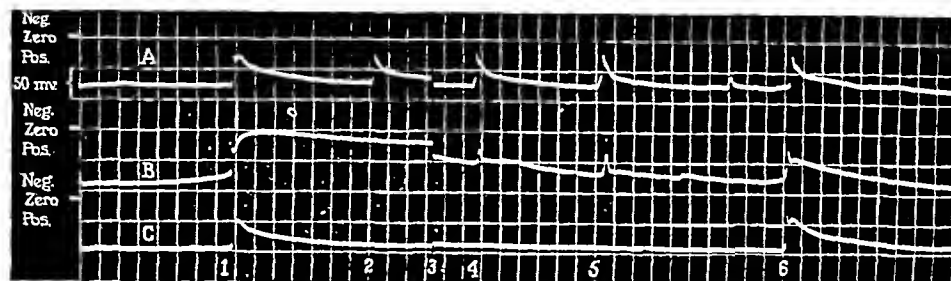


FIG. 1. Photographic record (employing a galvanometer with three strings) of an experiment arranged as in Fig. 2: 0.001  $M$  KCl at all points except at *B* where 0.001  $M$  KCl containing 1.5  $M$  ethyl alcohol is applied. Curves *A*, *B*, and *C* show the P.D. of *A*, *B*, and *C* with reference to *D* (which has been killed by chloroform before starting the experiment). The intervals between time marks represent 5 seconds.

The effect of alcohol on starting and stopping action currents is seen in Curve *B* (for description see page 611).

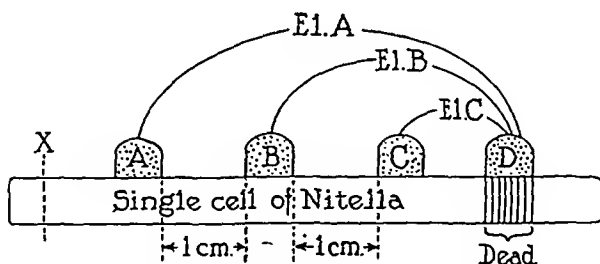


FIG. 2. Diagram to show the arrangement of an experiment: *D* is killed by the application of chloroform before the start of the experiment.

The cell is supported on a paraffin block, and is surrounded with air except where the contacts are applied.

which the alcohol was washed away by a stream of 0.001  $M$  KCl. Cotton soaked in 0.001  $M$  KCl was then replaced exactly on *B*: the record was set in motion, the camera was opened and at 4 the cell was stimulated electrically at *X*, producing a normal response at *A*, an

incomplete response at *B*, and none at *C*. Another stimulus at 5 had much the same result save that the response at *B* was greater. But the next stimulus (starting at *D*) at the spot marked 6 produced a much greater response: it will be noted that recovery has now made good progress and we see that the blocking effect of the alcohol has disappeared and that *A* now responds normally.

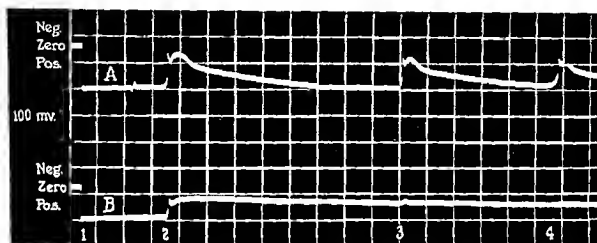


FIG. 3. Photographic record of an experiment arranged as in Fig. 2 with *C* omitted. At *A* and *D* 0.001 *M* KCl; at *B* 0.001 *M* KCl containing molar ethyl alcohol: Curves *A* and *B* show the p.d. of *A* and *B* with reference to *D* (which has been killed by chloroform before the start of the experiment). The intervals between time marks represent 5 seconds.

Curve *B* shows that in this case alcohol had no apparent effect until an action current arrived from another part of the cell (for description see below).

In many cases there was a larger degree of recovery at *B* after passage of an action current, and the cell could again be stimulated within 30 seconds. In such cases the recovery was less with each successive response, because of the loss of p.d., but the curve rose to practically the same level each time.

It sometimes happens that the application of alcohol produces no apparent effect until the cell is stimulated. This is illustrated by Fig. 3, the experiment being arranged as in Fig. 2 with *C* omitted. At the start *B* was 65 millivolts positive to *D* and this was not altered by the application of molar alcohol at the point marked 1. At the point marked 2 the cell was stimulated electrically at *X* and a normal response occurred at *A*. A response is also seen at *B* (where alcohol is applied) but in this case there is practically no recovery. The subsequent stimuli at 3 and 4 produced only slight responses at *B*.

As is to be expected, the effect of alcohol varies with the concentration. With 0.5 M alcohol there was no lowering of P.D. and irritability was normal. With molar alcohol, the P.D. slowly fell, the loss not exceeding 20 per cent in 5 minutes when there were no action currents. An action current arriving from another part of the cell sent the P.D. approximately to zero, and recovery of P.D. was slow and incomplete. After several action currents, recovery was absent and propagated variations were no longer transmitted, but a response at the spot in contact with alcohol could still be secured by cutting another part of the cell.<sup>4</sup> On removal of alcohol, and washing, the P.D. returned completely in about 5 minutes, irritability, responses, and transmission becoming normal.

The application of 1.5 M alcohol produced results like those shown in Fig. 1, the loss of P.D. taking place during from 5 to 100 seconds after the application of alcohol. During the loss of P.D. stimulation usually occurred after which the P.D. approached zero. There was little or no recovery and electrical stimulation at such a point as *X* (Fig. 1) produced no response at *B* but cutting at *X* produced a death wave at *B*. When the alcohol was removed complete irritability returned in from 3 to 15 minutes.

Alcohol at a concentration of 2 M stimulated in a few seconds: the P.D. fell to and remained at zero, irritability and transmission were abolished but a response at *B* (Fig. 2) could be secured by cutting at *X*. As in the other cases the action of alcohol was hastened by an action current arriving from another part of the cell. On removing the alcohol no recovery was observed during 10 minutes, but after 20 minutes the potential had risen to approximately the original value and irritability had returned.

The effects of 3 M alcohol were much like those of 2 M, except that after the removal of alcohol and washing the cell, there was only par-

<sup>4</sup>Cf. Osterhout, W. J. V., and Hill, S. E., *J. Gen. Physiol.*, 1930-31, 14, 473. The response consisted in a rise of the curve a little above zero and a gradual falling to zero. The stimulus of the cut causes the inner surface to lose its P.D. first, thus causing the curve to go above zero. This may be similar to the effect of cutting when the cell is in contact with 0.01 M KCl, as explained in former papers (Osterhout, W. J. V., and Harris, E. S., *J. Gen. Physiol.*, 1928-29, 12, 167) except that in the case of alcohol plus 0.001 M KCl the P.D.'s of each layer may be small.

tial recovery of potential, not to exceed 30 per cent, and no recovery of irritability. Usually the cell died within an hour or two.

The lowering of P.D. has been observed by Jost<sup>5</sup> in *Chara* and by Umrath<sup>6</sup> in *Nitella*.

In chemical stimulation repeated action currents are commonly observed<sup>7</sup> and it has been stated that this appears to depend on a rather sharp boundary of the stimulating agent which results in a rather steep electrical gradient in the neighborhood of the boundary. Unless this can be obtained we should not expect repeated action currents. In the case of alcohol a sharp boundary would not be expected since where alcohol and water meet a rather violent mixing occurs. This applies to a lesser extent when solutions of alcohol in water come in contact with water in the film covering the cell wall. It is therefore not surprising that we do not observe repeated action currents in our experiments.

It is most interesting to find that a non-electrolyte can reversibly alter P.D. to such an extent and it is important to discover how it is done. No doubt structural changes could account for it, e.g. by reversible coagulation or the formation of openings (as elsewhere discussed<sup>1</sup>), by changes in surface tension. On the other hand, alteration of the non-aqueous surfaces involving changes in the mobility of ions might bring it about. Alcohol might tend to dissolve out some of the non-aqueous constituents and thus change mobilities: it is probably more polar than the surface layers of the protoplasm and would therefore tend to increase their conductivity and the solubility of electrolytes in them.<sup>8</sup> Such changes might affect the inner and outer surfaces unequally, since these surfaces appear to differ. Further experiments will be undertaken to clear up some of these points.

Similar experiments on the sciatic nerve of the leopard frog (April)

<sup>5</sup> Jost, L., *Sitzungsber. Heidelberger Akad. Wissensch.*, Aht. B, 1927, Abhandl. 13, Nov.

<sup>6</sup> Umrath, K., *Protoplasma*, 1930, 9, 576.

<sup>7</sup> Osterhout, W. J. V., and Hill, S. E., *J. Gen. Physiol.*, 1929-30, 13, 459.

<sup>8</sup> Merely increasing the conductivity without changing relative mobilities would reduce the resistance of the protoplasm without changing the P.D., but the observed value of the P.D. might be slightly raised for reasons given elsewhere (Osterhout, W. J. V., and Harris, E. S., *J. Gen. Physiol.*, 1929-30, 12, 761).



gave negative results. The nerve was exposed near its origin by opening the dorsal body wall in the lumbar region: in this way injury to the nerve could be easily avoided. In some cases the nerve was exposed just above the knee. Concentrations of ethyl alcohol ranging from 1 to 5 molar (in Ringer's solution) were then applied without causing the leg muscles to twitch although subsequent electrical stimulation showed that the nerve was functioning normally.

This result does not seem to be due to the fact that the alcohol does not penetrate to the nerve fibres, for alcohol (3.5 M) applied to the nerve established a complete block in about 4 minutes without causing an action current. It is quite possible that the absence of action currents is due to the absence of a sharp boundary, as already suggested for *Nitella*.

Concentrations up to 5 M applied to the intact skin did not cause the characteristic wiping reaction which was, however, promptly elicited by acetic acid.

On the other hand it must be remembered that one can taste 1.5 M ethyl alcohol without difficulty.

Kemp and Waller<sup>9</sup> state that 5 to 10 per cent alcohol in saline gives a temporary contracture of the frog's sartorius when the muscle is immersed in the solution. This is evidently somewhat different from the negative variation observed in *Nitella*.

#### SUMMARY

Suitable concentrations of ethyl alcohol (1 to 1.5 M) applied to a spot on a cell of *Nitella* lower the P.D. enough to cause action currents. The alcohol then suppresses action currents arriving from other parts of the cell and acts as a block. After the alcohol is removed the normal P.D. and irritability return.

Similar experiments on the sciatic nerve and skin of the frog produced only a negative result.

<sup>9</sup>Kemp, H. P., and Waller, A. D., *J. Physiol.*, 1908, **37**, xliii (Proc.).

# TEMPERATURE CHARACTERISTIC FOR PRODUCTION OF CO<sub>2</sub> BY PHASEOLUS SEEDLINGS

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## I

A recent publication by Kurbatov and Leonov (1930) has endeavored to question the view that the velocities or frequencies of many processes in organisms submit to description in terms of a relatively simple and precise formulation of their relations to temperature as given by the expression  $\ln k \propto -\mu/RT$ . Specifically, they claim that the rate of respiratory production of CO<sub>2</sub> by seedlings of *Phaseolus aureus* (Roxb.) cannot be expressed as a function of temperature by the Arrhenius equation, and hence that the quantity  $\mu$ , "critical increment" or "temperature characteristic" (Crozier, 1924-25) is no constant. Their numerous experiments touch rather closely upon some with which we have been engaged for several years; hence it seems to us desirable to consider them with care, especially since the major error of several in the paper by Kurbatov and Leonov is one not infrequently encountered.

These authors protest that the production of CO<sub>2</sub> by a seedling is a "simpler" process than many activities of animals which have been considered from the standpoint of their precise relations to temperature. The grounds for this opinion are not given. In any particular case, however, "simplicity" within the terms of the problem as set for analysis must be decided by the evidence and not by vague forejudgments. This is apart from the fact that however "simple" the metabolic formation and release of CO<sub>2</sub> may be, chemically, it by no means follows that the experimental estimations of the rates of liberation of CO<sub>2</sub> by a group of growing seedlings are actually related in simple fashion to temperature, or to any other variable. We should point out here that Kurhatov and Leonov seem to be quite imperfectly ac-

quainted with the literature of this topic, even in the case of papers which they cite.<sup>1</sup>

## II

The experiments by Kurbatov and Leonov (1930) were made in the following way, which we must describe because the whole point of their argument in fact turns upon errors of technic:

Cylindrical vessels 30 cm. high and of 120 to 140 cc. capacity held 200 seedlings of *Phaseolus aureus* (Roxb.). These vessels were given 30 to 40 minutes for thermal adaptation in a thermostat, which the authors regarded as adequate; it is a simple matter to show that it is anything but adequate, as we shall later demonstrate. Moreover, unless the seedlings are stunted (*vide infra*) it is almost impossible to insert 200 seedlings of the plant into such a cylinder without injury due to packing and crowding. They give a temperature control of constancy "of the order of  $\pm 0.3^\circ$ ," which is easily demonstrated to be inadequate for precise work. The air stream was drawn through their cylinders at such a rate (2 to 2.5 liters per 40 minutes) that the atmosphere in the cylinder was renewed every 2 minutes; but the incoming stream passed first through a concentrated solution of NaOH and through a tower containing pumice moistened with concentrated NaOH solution. Hence the air in the respiration chambers must have been almost completely moisture-free; it is very difficult to maintain constancy of respiration with seedlings subject to progressive drying. In the paper by Kurbatov and Leonov no evidence is given that they sought at any time to ascertain if their seedlings gave a constant rate of excretion of CO<sub>2</sub> at constant temperature. We consider that these various kinds of technical inadequacy are probably more significant than the fact that their samples of CO<sub>2</sub>, collected in Ba(OH)<sub>2</sub> solution in Pettenkofer tubes, were not titrated until the next day, and that no details are given as to the method of storage, nor as to the method of titration.

## III

We have repeated, as exactly as possible, the essential procedure used in the experiments cited, but with certain very essential refinements.

<sup>1</sup> For example: they state (*loc cit.*, p. 160) that according to Crozier (1924-25; *cf.* also Navez, 1928-29)  $\mu$  is constant in the data of Kuijper (1910) on *Pisum* "from 0-26°," which is quite incorrect; so also is their statement of Hecht's (1918-19) results. They seem to be entirely innocent in their comments (Kurbatov and Leonov, 1930, p. 158) on methods of computing  $\mu$  from observations. Their related points dealing with the existence of critical temperatures (*loc. cit.*, p. 158, etc.) are so involved with misconceptions that we need only refer to the fact that they have been discussed some years ago (Crozier, 1924-25; 1925-26a, b; Crozier and Stier, 1926-27).

Seeds of *Phaseolus aureus* (Roxb.), answering the taxonomic criteria of the plants used by Kurhatov and Leonov, were obtained in a Chinese market store in Boston. These had been imported from China; those used by the Russian workers came initially from Bokhara. The plants were grown in sterile maple sawdust; the sawdust had been repeatedly treated with NaOH or KOH, then with HCl, then washed with boiling water, and subsequently boiled several times or autoclaved, and well soaked in water. Seeds were found to swell in 12 to 18 hours, and germinated in 24 hours. The seedlings were very uniform as to size and general appearance. They were grown for 3 to 10 days in a thermostat at 23°. Even after transference to the respiration chamber, they remained free from moulds throughout the duration of the observations.

The respiration chamber was a Pyrex cylinder 32.5 cm. long, 2.5 cm. diameter, of 140 cc. capacity (Fig. 1). Three laterally placed tubes were arranged for the insertion of thermocouples. The chamber was well washed in sterile water before each experiment.

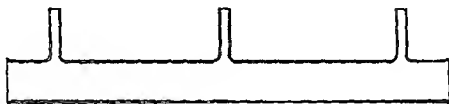


FIG. 1. Respiration chamber, with side arms for introduction of thermocouples.

The seedlings were removed from the sawdust beds, kept exactly vertical (*cf.* Navez, 1928-29; Navez and Crozier, 1929), washed rapidly with distilled water, and put vertically in the respiration chamber. With seedlings of this sort it is possible to place 80 to 120 in the chamber without obvious mechanical injury or excessive crowding—although this general procedure is certainly not one to be recommended. Each end of the chamber was closed by a heavy rubber stopper. The air inlet was at the bottom, the outlet at the top.

A current of air was drawn from outside the building (avoiding traces of illuminating gas) by a water pump, passed through a tube of cotton wool, dilute  $H_2SO_4$ , 120 cm.  $\pm$  of soda-lime, 3 wash bottles of concentrated NaOH, 1 or 2 towers of distilled water with glass beads, and then to the respiration chamber. The wash bottles and saturating columns were in the thermostat. The rate of flow of the moist  $CO_2$ -free air was 3 liters per hour.

The thermostat (*cf.* Crozier and Stier, 1926-27) could be adjusted at any temperature between 0° and 30° (or above), and maintained a constant temperature to within 0.01°C. It was located in a dark-room, and itself covered to exclude chance illumination. The plants were not illuminated during an experiment. Tests showed that carrying out the preliminary manipulation of the seeds in dim red light did not influence the results in any way.

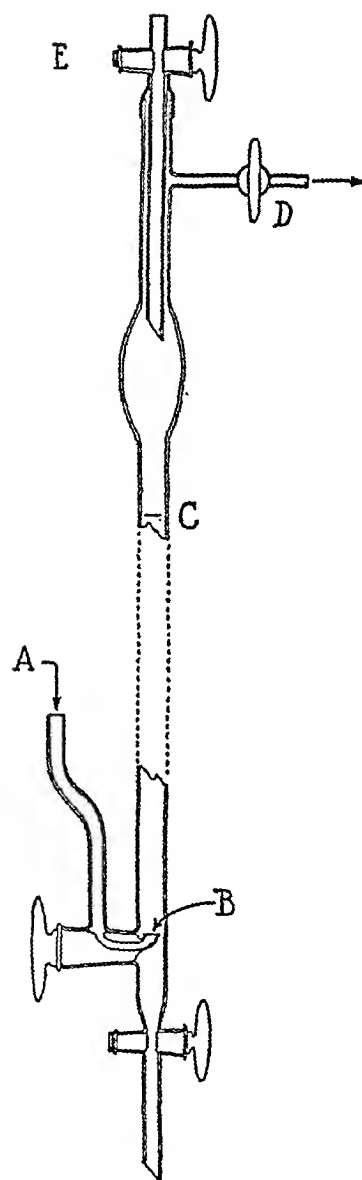


FIG. 2. Tube for absorption of CO<sub>2</sub> in baryta water. Air from respiration chamber enters at *A*; the inlet orifice at *B* is covered by a thin platinum plate, sealed on, and perforated with many small pin-prick apertures; the upper level of the baryta water is at the mark *C*; the 3-way stop-cock at *D* connects with a soda-lime tube, or air is aspirated as indicated by the arrow; *E* is a stop-cock for filling from a reservoir of baryta solution. Scale equals  $\frac{1}{2}$  linear.

CO<sub>2</sub> excreted was absorbed in Ba(OH)<sub>2</sub> solution (0.064 N to 0.072 N, in different cases), using a Pettenkofer tube of special design (Fig. 2). Titrations were made in triplicate within 60 minutes after an absorption period was completed, with special care to exclude CO<sub>2</sub>, using 0.088 N to 0.047 N HCl in a sensitive burette, with phenolphthalein (2 to 5 drops to 10 to 20 cc. of haryta water). Color matching was done under a daylight lamp by comparison with the same amounts of neutralized haryta water and indicator in an identical flask.

TABLE I

Results in two experiments in which the CO<sub>2</sub> production by seedlings of *Phaseolus aureus* was estimated under conditions approximating those employed by Kurbatov and Leonov (1930). In Experiment A, 200 seedlings 3 days after sprouting (dry weight 3.850 gm.) were placed in the respiration tube, at temperature 18.30°; in Experiment B, 190 seedlings 5 days after sprouting (dry weight 4.508 gm.) were employed at temperature 18.15°. Drying was avoided by maintaining an air stream of constant humidity. The figures for production of CO<sub>2</sub> are averages of concordant triplicate determinations. See text.

| A                                |   | B                                |   |
|----------------------------------|---|----------------------------------|---|
| 200 seedlings<br>Temp., 18.30°   |   | 190 seedlings<br>Temp., 18.15°   |   |
| Successive periods of<br>45 min. | Mg. CO <sub>2</sub> produced per<br>45 min. | Successive periods of<br>45 min. | Mg. CO <sub>2</sub> produced per<br>45 min. |
| 1                                | 11.30                                       | 1                                | 13.45                                       |
| 2                                | 14.80                                       | 2                                | 11.85                                       |
| 3                                | 20.50                                       | 3                                | 12.70                                       |
| 4                                | 16.45                                       | 4                                | 13.40                                       |
| 5                                | 18.00                                       | 5                                | 18.05                                       |
| 6                                | 14.60                                       | 6                                | 14.60                                       |
| 7                                | 15.60                                       | 7                                | 13.35                                       |
| 8                                | 16.25                                       | 8                                | 15.10                                       |
| 9                                | 15.70                                       | 9                                | 13.40                                       |

## IV

1. When as many as 200 seedlings are in the respiration chamber it is impossible to obtain constancy in the rate of evolution of CO<sub>2</sub> at constant temperature. Seedlings of the proper age (usually 3 to 6 days) and small enough for this purpose were grown at about 22-23°. When too many are crowded into the chamber, geotropic and thigmotactic excitation are inevitable, and injury as well. An experiment of this sort is summarized in Table I. The deviations from constancy shown in such experiments are of the order of magnitude exhibited by

TABLE II

A. 100 seedlings, 6 days old; temp. = 18.70° for 1.25 hrs., before determinations of rate of production of CO<sub>2</sub> began; figures are averages of triplicate titrations

| Successive intervals of 45 min. | Mg. CO <sub>2</sub> per 45 min. |
|---------------------------------|---------------------------------|
| 1                               | 6.94                            |
| 2                               | 6.87                            |
| 3                               | 7.05                            |
| 4                               | 7.20                            |

The temperature was now lowered to 15.00° (within 9 min.), kept at this level for 50 min., and during the next 16 min. it was raised to 18.70° again.

|   |      |
|---|------|
| 5 | 4.55 |
| 6 | 7.05 |
| 7 | 7.25 |

B. 120 seedlings 5.5 days old; temp. = 18.70° for 1.20 hrs. before determinations of CO<sub>2</sub> began

| Successive intervals of 45 min. | Mg. CO <sub>2</sub> per 45 min. |
|---------------------------------|---------------------------------|
| 1                               | 7.45                            |
| 2                               | 7.45                            |
| 3                               | 7.55                            |
| 4                               | 7.45                            |

The temp. was now raised to 22.8°, maintained for 60 min., during the next 15 min. the temp. was brought back to 18.70°.

|   |      |
|---|------|
| 5 | 5.48 |
| 6 | 7.71 |
| 7 | 7.45 |

C. 60 seedlings, 14 days after sprouting; 2 hrs. thermal adaptation at 18.7°

| Successive intervals of 45 min. | Mg. CO <sub>2</sub> per 45 min. |
|---------------------------------|---------------------------------|
| 1                               | 7.64                            |
| 2                               | 7.35                            |
| 3                               | 7.64                            |

The temp. was then changed during 12 min. to 14.5°; 35 min. allowed for thermal adaptation.

|   |      |
|---|------|
| 4 | 6.58 |
| 5 | 4.97 |
| 6 | 5.13 |
| 7 | 5.08 |

TABLE II—*Concluded*

| D. 120 seedlings 4.5 days after sprouting; allowed 1.5 hrs. for thermal adaptation at 18.7°      |                                 |
|--|---------------------------------|
| Successive intervals of 45 min.  | Mg. CO <sub>2</sub> per 45 min. |
| 1  | 4.48                            |
| 2  | 4.42                            |
| 3  | 4.44                            |
| The temp. was then lowered during 13 min. to 16.1°; 35 min. allowed for adaptation.              |                                 |
| 4  | 3.80                            |
| 5  | 3.48                            |
| 6  | 3.52                            |
| 7  | 3.48                            |
| 8  | 3.56                            |
| The temp. was brought back again to 18.7° during 18 min; 35 min. allowed for thermal adaptation. |                                 |
| 9  | 4.29                            |
| 10   | 4.62                            |
| 11   | 4.49                            |
| 12   | 4.435                           |
| After an interval of 12 hrs. at 18.7°, 3 successive determinations gave the following:           |                                 |
| 13   | 4.48                            |
| 14   | 4.46                            |
| 15   | 4.56                            |

successive determinations at the same temperature in the experiments by Kurbatov and Leonov, amounting to as much as 40 per cent, even though most of their "return" observations involve temperatures above 20°, which we find to be an upper critical temperature for these seedlings.

2. When the temperature is changed, a further complication enters. This we may illustrate by means of experiments in which the ill effects of crowding were avoided, and in which a satisfactory degree of constancy is obtained for the rate of respiration at constant temperature. The point is that in their experiments Kurbatov and Leonov (*loc. cit.*, p. 148) proceeded as follows: taking account of the fact that thermal adaptation of potato tubers and the like is "very slow," and that one hour has been found sufficient for the thermal adaptation of seedlings of *Pisum*, 30 to 40 minutes were allowed for thermal adaptation of



*Phaseolus* which are only about one-seventh the weight of a *Pisum* seedling; no tests were made, apparently, to demonstrate the efficacy of this procedure. Results such as those given in Table II plainly show that the immediate effect of a change of temperature, at least within the range which interests us, is to produce a lessened apparent rate of excretion of CO<sub>2</sub> for some time thereafter, if the temperature has been raised, or even in some instances if the temperature has been *lowered*, although in the latter case the apparent rate of excretion of

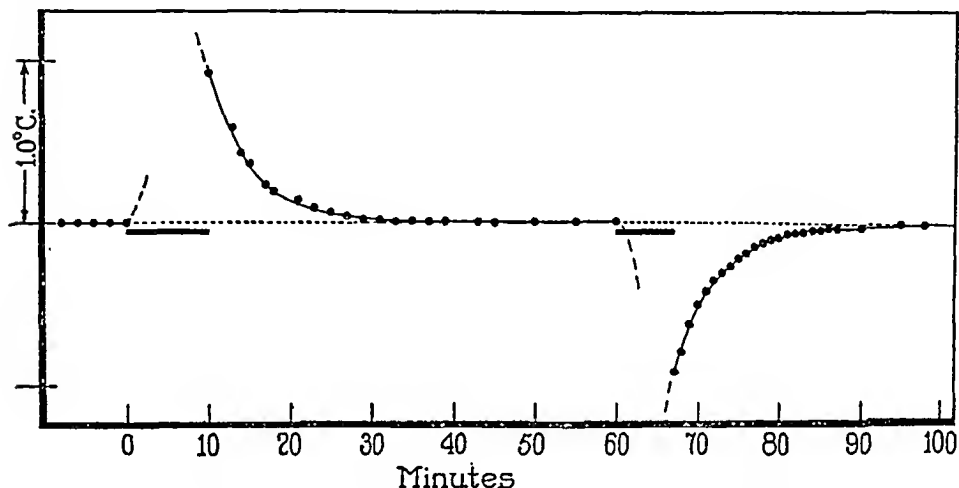


FIG. 3. Curves of thermal adjustment of the respiration chamber after a change of temperature in the thermostat. The temperature was at first, in the case illustrated, 16.25°C.; after 1.5 hours, at this temperature, the temperature of the thermostat was lowered to 11.50° during 10 minutes (represented by horizontal bar). Complete thermal equilibration was obtained in approximately 20 minutes.

The new temperature level was maintained for about 30 minutes, the temperature then being raised, during 7 minutes, to 17.70°; the readjustment of thermal balance was about complete in 20 to 25 minutes.

CO<sub>2</sub> is below that which is a steady rate for the new (lower) temperature. The effect is magnified, in other experiments, by crowding. It is not primarily due to failure of the interior of the respiration chamber to assume quickly enough the temperature of the thermostat. We are not concerned now to account for this; because of the complex circumstances attending the use of many individuals crowded into a respiration chamber we are not called upon to account for the effect in terms of an intrinsic "response" of the plant tissues, such as might

influence the mere rate of physical exit of  $\text{CO}_2$  from the surfaces of the seedlings. We are interested only in the demonstration that 30 to 40 minutes is altogether too short an interval for thermal adaptation; this is equally apparent in tests with single seeds. We may note also (Table II) that there is a distinct tendency for the reading succeeding the "abnormal" one just subsequent to a change of temperature, to be too high.

To study the rate of thermal adjustment in the respiration chamber after a change of temperature, a thermopile was inserted in the middle side-arm of the chamber. The thermopile was made of four copper-constantan couples, and located at the axis of the respiration tube; the other junction was placed in the thermostat at about 5 cm. away from the tube. The circuit was closed through a short-circuiting key and low-resistance galvanometer having a resistance of the same order as the thermojunctions (59.9 ohms). The maximum variation in the temperature of the thermostat was  $\pm 0.005^\circ\text{C}$ . After a period of at least  $\frac{1}{2}$  hour of thermal equilibrium between the respiration chamber and the thermostatic bath, i.e., of a temperature difference not greater than  $0.005^\circ$ , the temperature of the thermostat was changed as rapidly as possible (7 to 10 minutes), and observation made of the curve of thermal adjustment of the respiration chamber (cf. Fig. 3). Tests of this sort demonstrate that complete thermal equilibrium is reached after approximately 10 minutes, following a change of temperature of  $2^\circ$ . It was also shown in this way that the current of air delivered to the respiration chamber was in thermal equilibrium with the thermostat, and that the heat capacity of the plants packed in the respiration chamber was not very high when compared with that of water.

Since the establishment of a steady state of production of  $\text{CO}_2$  requires at least 45 to 60 minutes after a change of temperature of about  $2^\circ$ , this behavior cannot be primarily a thermal effect, but must be due to internal causes. The examination of the kinetics of such effects may be undertaken profitably at a later date.

## V

When conditions are arranged in such a way as to avoid unreasonable crowding, and so far as possible to eliminate geotropic and other excitation, and when adequate periods of thermal adaptation are permitted, the relation of the rate of elimination of  $\text{CO}_2$  by *Phaseolus aureus* (Roxb.) is quite different from that described by Kurbatov and Leonov (*loc. cit.*). When their conditions are followed, however, so far as possible, the kind of result given at length in their paper is easily secured. The high increments ( $\mu$ ) occasionally indicated in such series are markedly influenced by several sources of confusion already

mentioned. (We should point out, however, that below about 12° the delay in thermal adaptation is not so pronounced as above this temperature.)

When proper allowance for thermal adaptation is made, the result is as given in Tables III and IV (Fig. 4).

TABLE III

80 seedlings, 9 days old; 60 min. allowed for thermal adaptation after each change of temperature; steady state assured by successive determinations.

| Temp. | $\frac{\text{mg. CO}_2}{45 \text{ min.}}$ | $\frac{\text{mg. CO}_2}{45 \text{ min.}}$ calc. from<br>$\mu = 16,500$ | $\Delta$ |
|-------|---|--|----------|
| °C.   |   |  |          |
| 18.0  | 5.570                                     | 5.615  | +0.045   |
| 16.1  | 4.525                                     | 4.655  | +0.130   |
| 12.2  | 3.220                                     | 3.155  | -0.065   |
| 20.3  | 7.130                                     | 7.033  | -0.097   |

TABLE IV

80 seedlings, 7 days after sowing (dry weight 1.644 gm.); 65 minutes allowed for adaptation at each change of temperature.

| Temp. | $\frac{\text{mg. CO}_2}{45 \text{ min.}}$ | $\frac{\text{mg. CO}_2}{45 \text{ min.}}$ calc. from<br>$\mu = 16,500$ | $\Delta$ |
|-------|---|--|----------|
| °C.   |   |  |          |
| 12.4  | 2.410                                     | 2.405  | -0.005   |
| 13.4  | 2.610                                     | 2.660  | +0.050   |
| 16.8  | 4.075                                     | 3.730  | -0.345   |
| 18.1  | 4.110                                     | 4.236  | +0.126   |
| 19.8  | 4.860                                     | 4.978  | +0.118   |
| 20.2  | 5.395                                     | 5.190  | -0.205   |
| 21.1  | 5.370                                     | 5.610  | +0.260   |
| 23.2  | 5.650                                     |  |          |

The data of Tables III and IV, and the measurements from two other experiments, are plotted in Fig. 4. Since we are interested in the rates of increase of ( $\Delta \text{CO}_2 / \Delta t$ ) with increase of temperature, we are at liberty to multiply all the observed rates in any one series of determinations by a constant which (Fig. 4) brings the several sets of measurements into position for comparison. Between 12° and 20-21°

the data are satisfactorily described by the equation  $\log(\Delta \text{CO}_2/\Delta t) \approx -\frac{\mu}{TR} + C$ , and from the slope of the line drawn through them  $\mu \approx 16,500$  calories. The *calculated* values for mg.  $\text{CO}_2$  per 45 minutes, given in Tables III and IV, are computed with this value of  $\mu$ . The agreement is satisfactory. The extreme deviations of duplicate and

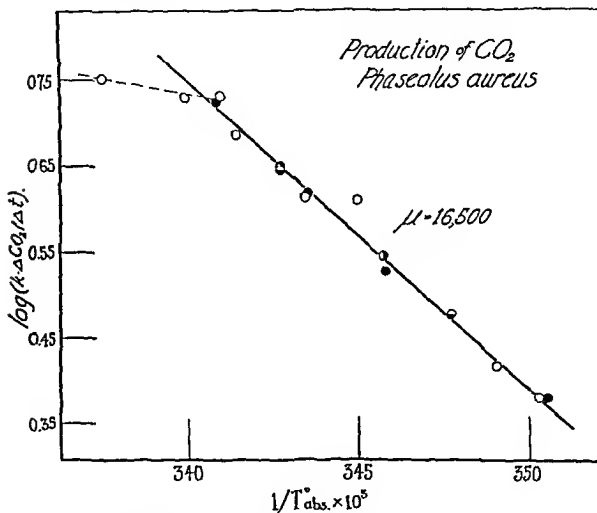


FIG 4. Rate of production of  $\text{CO}_2$  as related to temperature; four series of measurements. See text.

triplicate determinations of  $(\Delta \text{CO}_2/\Delta t)$  average in our data 5.4 per cent of the respective means; in the best series the latitude of dispersion is 1.5 per cent. This is greater than the deviations of the observed from the calculated values of  $(\text{mg. CO}_2)/(45 \text{ minutes})$  given in Tables III and IV, which average 3.5 per cent of the respective means. The single extreme deviation, clearly due to an uncontrolled "error," is less than 10 per cent (point at  $16.8^\circ$ ). There is definite evidence of a

critical temperature at 20–21° (Fig. 4). This is equally clear in Fig. 5, where the rates are plotted against  $t^{\circ}\text{C}$ . It is of interest that in seedlings of related plants (*Pisum*; *Vicia*, Navez, 1928–29; *Lupinus*, and in *Zea*, Tang, 1930–31) the same critical temperature is found. A greater number of determinations at different temperatures is of course desirable, but the temperature characteristic is better established by a different technic.

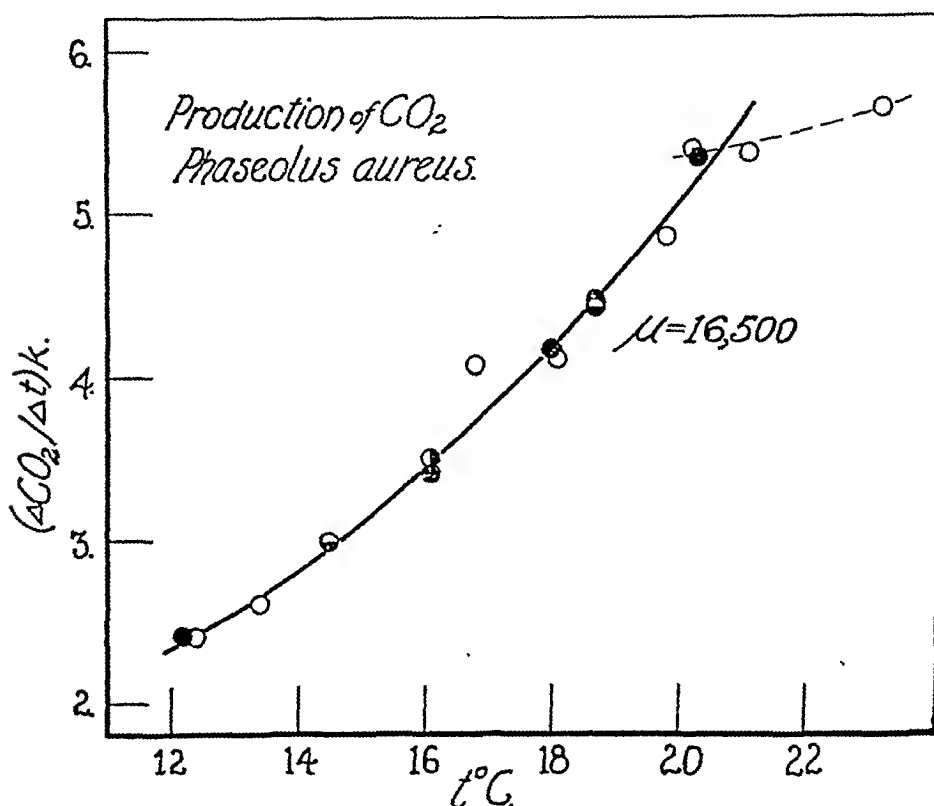


FIG. 5. Data of Fig. 3, with rate of excretion of CO<sub>2</sub> plotted against  $t^{\circ}\text{C}$ .

We do not regard this method of experimentation as really adequate for the purpose,<sup>2</sup> but with reasonable care in its use, it does enable one

<sup>2</sup> It should be mentioned especially that when *single* seedlings are studied, by an appropriate technic, there is found a slight but steady increase in the rate of respiration with time. The method here used does not permit taking this into account. In later publications certain of these details will be given. (Cf. Tang, *J. Gen. Physiol.*, 1930–31, 14, 631.)

to show (1) that the rate of excretion of  $\text{CO}_2$  is proportional to the exponential of  $-\mu/RT$ , and (2) that the value of the temperature characteristic  $\mu$  agrees quantitatively with that obtained from other similar cases (cf. Crozier, 1924-25; 1924; Navez, 1928-29), and with data derived from technically superior experiments with single seedlings.

#### SUMMARY

The temperature characteristic for respiratory production of  $\text{CO}_2$  by young seedlings of *Phaseolus aureus* (Roxb.) is  $\mu = 16,500$  calories,  $12-21^\circ\text{C}$ ., even when the analyses depend upon the use of many seedlings crowded in a small respiration chamber, provided reasonable precautions are taken to avoid injury and to permit proper thermal adaptation. There is evidence of a definite critical temperature at  $20-21^\circ$ . These findings agree quantitatively with those obtained with other similar seedlings, and contradict the results reported by Kurbatov and Leonov (1930); the reasons for this are analysed.

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# TEMPERATURE CHARACTERISTICS FOR THE OXYGEN CONSUMPTION OF GERMINATING SEEDS OF LUPINUS ALBUS AND ZEA MAYS

By PEI-SUNG TANG

(From the Laboratory of General Physiology, Harvard University, Cambridge)

(Accepted for publication, March 28, 1931)

## I

Although the respiration of germinating seeds considered as a function of temperature has often been studied, actual data which are accurate enough for quantitative treatment of the relationship between respiration and temperature are very scanty. The few instances which can be subjected to such a treatment were brought together by Crozier (1924), who called attention to the repeated occurrence of temperature characteristics ( $\mu$ ) of certain magnitudes, and to the incidence of sharp breaks in the curves at certain temperatures (cf. also Navez, 1928-29). These appear clearly when the logarithms of the rates of respiration are plotted against the reciprocal of the absolute temperatures according to the Arrhenius equation, but are equally evident in other forms of representation. Above and below such a break, the values of the temperature characteristic are different. Since the publication of that paper little additional data have been made available on this particular point. The present paper considers the temperature characteristics for the oxygen consumption of the germinating seeds of *Lupinus albus* and of *Zea mays*; the observations were undertaken as part of a more extensive investigation (cf. Crozier, Stier, and Pincus, 1929), of which further portions will be described subsequently.

## II

Microrespirometers of the type described by Warburg (1926) were used in this study. The thermostat was so controlled that any temperature from above 0°C. to 50°C. could be maintained to within  $\pm 0.005^\circ$  for a long time (cf. Crozier and





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ture to the first reading at the next temperature, a period of 30 minutes elapsed, about 5 minutes being used for the change of temperature, usually in a step of 2 degrees. During the change and the subsequent period of adaptation, the vessels and the seeds remained undisturbed, and the stop-cocks on the manometers were opened so as to connect the vessel with the outside air.

Light was not entirely excluded from the seeds. Diffuse light from the laboratory window and lamps was not avoided. But since there was no formation of chlorophyll during the course of an experiment photosynthesis did not play a part here, and it is unlikely that the fairly constant diffuse light has much to do with respiration directly, though this yet remains to be investigated.

### III

Fig. 1 shows the data for *Lupinus albus*. The figures from several experiments were brought together by a conversion factor, using as standard (100 per cent) the readings at 13° in the upper group, and 22° in the lower group. Consequently the single point at these respective temperatures represents several readings.<sup>1</sup> The first five readings in the upper group, denoted by open circles, taken at 18°, 16°, 14°, 12°, and 10° respectively, were given no weight in these experiments because during this time there occurs a change of the rate of oxygen consumption of the seeds with time, aside from the question of thermal adaptation to be discussed later. Below 20° the points fall very consistently in a band the slope of which gives the temperature characteristic  $\mu = 16,600$ . In both groups a break occurs at about 19.5°, and above that temperature the points fall into another band the slope of which is  $\mu = 11,700$ . In both groups the extent of scatter in the first band is  $\pm 5$  per cent, while it is  $\pm 12$  per cent in the band above 20°, as drawn, but may be smaller in single instances, for it appears in many

<sup>1</sup> Preliminary experiments showed that we cannot compare the absolute values of oxygen uptake, expressed either as per individual seed, or in terms of unit weight, or unit surface, or of unit volume; hence the averaging of observations from different seeds can be done legitimately only after the rates have been adjusted empirically to a common basis. In Figs. 1 and 2 this has been done by taking the rate of respiration of one seedling at a given temperature (13° or 22°C.) as 100 per cent, and multiplying the rates for the other seeds by factors. This has the effect of bringing into identical position the lines fitted to the data for the individual series of measurements, without in any way changing their slopes. When this has been done, the corrected rates (not their logarithms) can be averaged with some confidence that one is averaging only the chance or fluctuating variations to which the measurements are subject.

Stier, 1927). The manometer vessels used are conical in shape, with capacities of about 13 cc., provided with a side arm, and with a cylindrical inset about 15 mm. high and 8 mm. in diameter. Each vessel is filled with 5 cc. of 10 per cent KOH solution through the side arm and a portion of the liquid is pipetted into the inset to fill the latter to about a fifth of its height, care being taken not to touch the wall of the inset with KOH. A seed is placed on top of the inset with the hypocotyl or radicle pointing downward near the center of the cylinder, not touching the wall. Ample space is provided for gas exchange between the inset and the vessel proper.

The manometers are not shaken during the course of the experiment, thus avoiding geotropic stimulation (*cf.* Navez, 1928-29; Navez and Crozier, 1929; in *Ms.*) and other complications resulting from shaking. Preliminary experiments showed that the readings taken in this way do not differ from those taken when the gas within the vessel is stirred by rapid raising and lowering of the solution in the manometer at frequent intervals throughout the course of the experiment. The fact that the seed is placed above the absorbing KOH, in air, so that the diffusion of oxygen into the seed is direct, and the fact that CO<sub>2</sub> is heavier than air, tending to descend from the seed to the KOH below, justify the elimination of shaking. Phenolphthalein tests at the end of an experiment show that the amount of KOH used both in the inset and in the vessel proper is in excess of the CO<sub>2</sub> produced, and that there is no creeping of the alkali up the wall of the inset. Since the seed is changing in volume during the time when it is in the vessel, there is a slight change of the apparent "vessel constant." But this was found to be negligible, being not more than 1 per cent.

Two kinds of seeds were used in the experiment: *Lupinus albus* and *Zea mays*. The former was obtained from a seed store; the latter was kindly supplied by the Department of Plant Breeding of the University of Minnesota, and is known as "Culture 16, Inbred from Rustler, 9 years selfed."

The seeds were germinated in moist maple sawdust which had previously been repeatedly boiled in distilled water. They were left in a dark incubator at 23° ± 0.5 until the seed coat was broken and the hypocotyl or the radicle was about a millimeter long. This in the case of *Lupinus* required only 12 hours; in the case of *Zea*, 36 hours. No fungus growth was observed on the seeds.

When about to start an experiment, the seeds were gently taken out and cleared of any adhering sawdust, then carefully transferred to the vessel, and seated on top of the inset in the position already described. For the transfer a pair of forceps was used, and as far as practicable stimulation of the seed was excluded. The vessel was then mounted on the manometer and placed in the thermostat in the customary manner. Six to seven respirometers were employed in each series of experiments, one of which served as a baro-thermometer. After half an hour of adaptation, the stop-cocks of the manometers were closed and the first readings were taken. Readings were taken at 15 minute intervals thereafter, and at the end of 1 hour the temperature was changed, the seeds being allowed to adapt for about 25 minutes before further readings were taken. From the last reading at one tempera-

ture to the first reading at the next temperature, a period of 30 minutes elapsed, about 5 minutes being used for the change of temperature, usually in a step of 2 degrees. During the change and the subsequent period of adaptation, the vessels and the seeds remained undisturbed, and the stop-cocks on the manometers were opened so as to connect the vessel with the outside air.

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cases (cf. Crozier and Stier, 1925-26; 1926-27; Pincus, 1930-31) that there is an abrupt shift in the absolute magnitude accompanying a change in the slope of the curve beyond a critical temperature. This absolute shift is sometimes to a level lower and sometimes to a level higher than the magnitude beyond the critical temperature. When

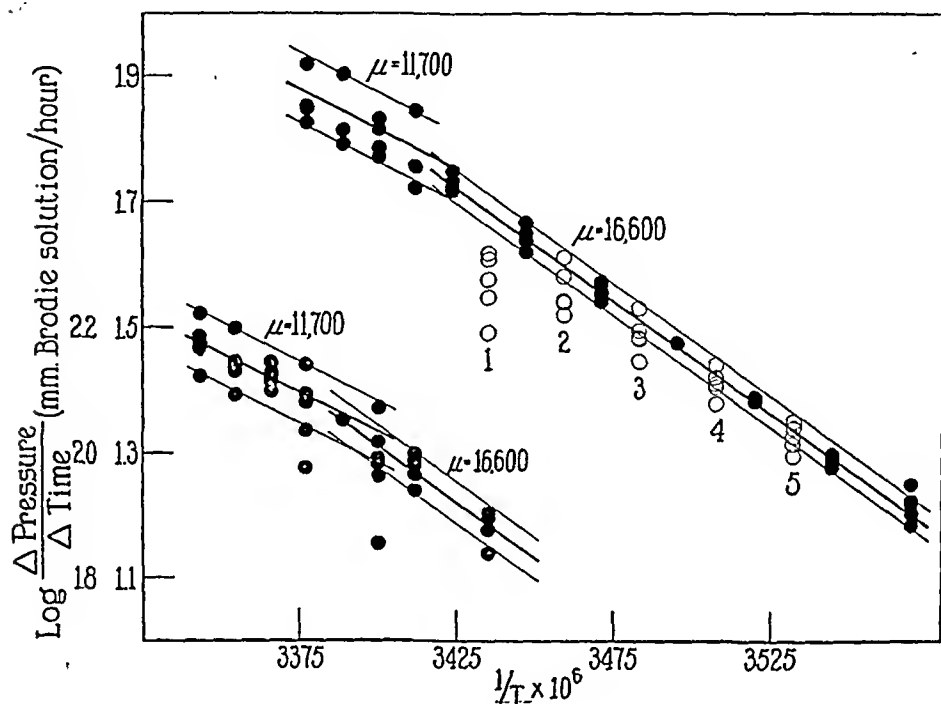


FIG. 1. Mass plot of data for *Lupinus albus*. The several experiments are grouped together by multiplication by a factor for each set, using one of the readings at 13° (upper group) or 22° (lower group) as the standard (100 per cent). The ordinate represents the logarithm of the change of pressure (in mm. of Brodie solution) per hour; the change in pressure is proportional to the volume of oxygen consumed. The first five readings in the upper group, numbered chronologically and marked with open circles, are given no weight, as explained in the text.

the observations are grouped together by the use of a single factor for the data on both sides of the critical temperature the range of variation may thus be magnified on one branch of the plot. Besides this question of an abrupt change in the absolute magnitude of the rates, there is also the possibility that the intrinsic latitude of variation may be different on the two sides of the critical temperature (cf., e.g., Crozier

and Stier, 1926-27). It is impossible to differentiate between the two in the present case, and probably both factors come into effect.

It may be of interest to note that if the temperature changes are made in a way such as to pass from 18° to higher temperatures, as is represented in the lower group of points in Fig. 1, the readings fall consistently in two straight bands and the "lag" which was encountered in the upper curve is not apparent. Two points which belong to

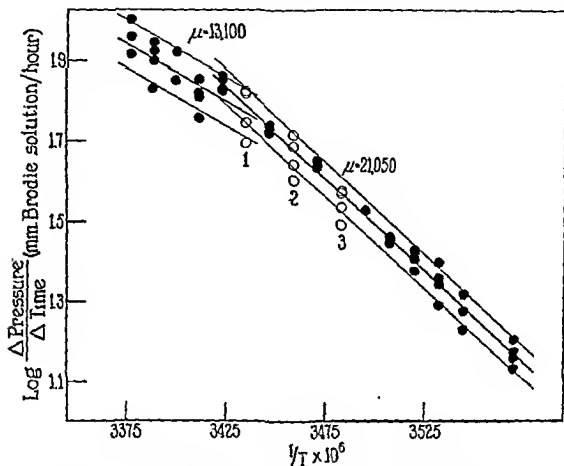


FIG. 2. Mass plot for observations on *Zea mays*. The method of presentation is similar to that used in Fig. 1. The first three readings in open circles and numbered chronologically, are given no weight, as explained in the text.

one individual seedling fell far below the lower boundary of the band (Fig. 1, lower group). There is reason to suppose that this seedling was injured during the course of the experiment.

Fig. 2 exhibits the data upon the oxygen consumption of *Zea mays*. Discounting the first three readings, taken at 18°, 16°, and 14°, as in the previous case, the points again fall within two bands, with the break occurring at 19.5°. The band below that temperature has a



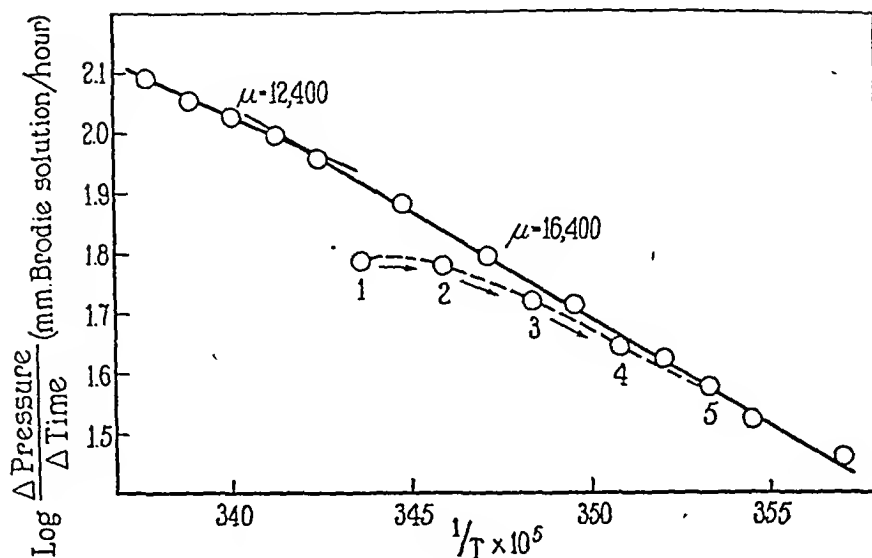


FIG. 3. A typical experiment with *Lupinus albus*; note the deviation of the first five readings from the straight line (see text).

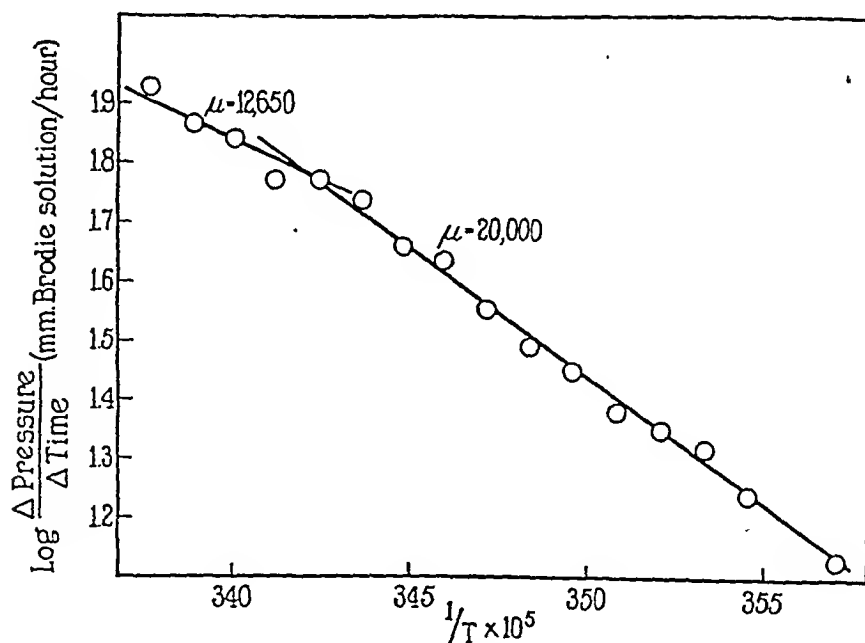


FIG. 4. A typical experiment with *Zea mays*; note the absence of any deviation in this particular experiment. (In other individual seedlings there was a drift with time during the first four readings.)

slope of  $\mu = 21,050$ , with a scatter of  $\pm 10$  per cent, and the band above  $20^\circ$  has a slope of 13,100 with about the same latitude of variation. One representative experiment from each of the two mass plots in Figs. 1 and 2 is given in Figs. 3 and 4.

A series of experiments revealed the fact that the rate of respiration of both *Lupinus* and of *Zea* seedlings changes with time during the first few hours of the experiment, under these conditions, gradually

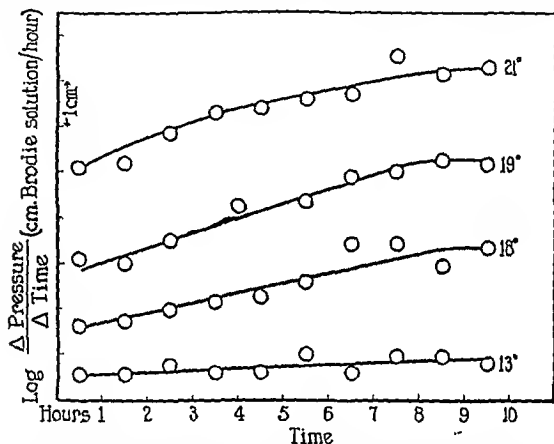


FIG. 5. The rate of oxygen consumption of *Lupinus albus* at different temperatures, plotted against time. The first reading was taken about  $1\frac{1}{2}$  hours after the seeds were placed in the vessels.

reaching a constant level. This effect is more pronounced in the case of *Lupinus* than in *Zea*, and is a function of temperature. The change of rate of respiration is more rapid the higher the temperature, and the flat portion of the time curve is reached sooner. Thus, for *Lupinus* this level was reached in 8 hours at  $18^\circ$ , in 7 hours at  $19^\circ$ , and in 6 hours at  $21^\circ$ . At  $13^\circ$  the rate changes so slowly that for practical purposes it may be considered as constant. These effects are illustrated in Fig. 5. The same general relation holds for *Zea*, only to a less

marked extent. It was considered quite unusual that such a change of rate should occur, for the work of various authors (*e.g.*, Warburg, 1926; Navez, 1928–29) on the respiration of both animals and plants indicates that the rate of respiration is constant with respect to time at least within short durations. That the effect is not due to lack of thermal adaptation is seen from the fact that except after the first few

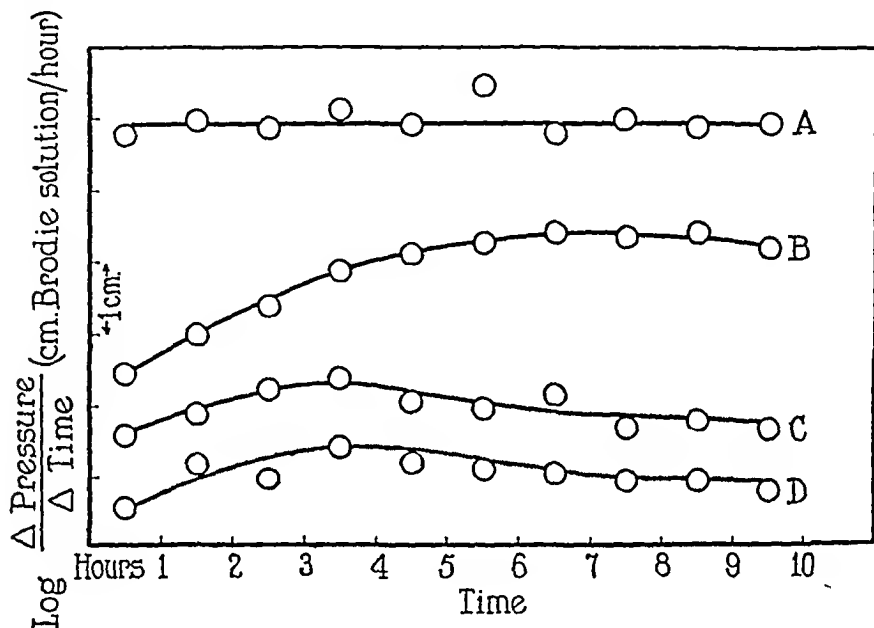


FIG. 6. The rate of oxygen consumption of *Lupinus albus* seeds which had been subjected to different conditions of germination, plotted against time, all at 18°. Curves A and B represent rates for seeds germinated on moist sawdust and transferred to the respiration vessels; A is 9 hours older than B at the time of transfer to the respirometer and the beginning of observations. C represents rates for seeds germinated over water, in a moist chamber, and then transferred to the respiration vessel. D represents rates for seeds germinated in the vessel over the alkali, without transfer. B, C, and D are similar in age, and are 9 hours younger than A.

readings the points on the graphs of Figs. 1 and 2 fall consistently on a straight line. Furthermore, a period of at least  $1\frac{1}{2}$  hour elapsed before the first readings in Fig. 5 were taken, and that length of time ought to have been enough for the small vessel and its contents to come to temperature equilibrium. The most probable reason for the occurrence of such an effect is that it is due to a change in the condition of the seed

when transferred from the moist sawdust, with which it was in direct contact and in which its hypocotyl was surrounded by a film of liquid water, to the respiration vessel in which it is only surrounded by a moist atmosphere and in which the hypocotyl is entirely free from direct contact with any liquid or solid. Experiments in progress substantiate this explanation. In such experiments the seeds were germinated over water in a moist chamber, (C, Fig. 6) or were germinated directly in the manometer vessel over the alkali (D, Fig. 6). These seeds behave quite differently from the ones which were germinated on the sawdust (B, Fig. 6), though all other conditions were maintained the same. They show a very slight rise in the rate of respiration during the first 3 hours at 18°. This is probably due to processes accompanying the initial stage of germination. The rate then gradually arrives at a steady value, after about the fourth hour. Thus the "time effect" seems to be due mainly to mechanical and perhaps other disturbances due to transferring the seeds from the germinator to the respiration chamber, and the initial increase in the rate of respiration is therefore an expression of response or reaction, similar to the increased rate of production of CO<sub>2</sub> in the geotropic response of *Vicia* seedlings, (Navez, 1928-29; Navez and Crozier, 1929). This "stimulating" effect is apparent only when the seeds are still in the stage when the hypocotyl is just emerging from the seed coat. Experiments made with seeds germinated on sawdust for 9 hours longer than usual, exhibit a constant rate of respiration with time (A, Fig. 6).

#### IV

From these results it appears that the temperature characteristics for oxygen consumption by the germinating seeds of *Lupinus albus* are 16,600 below 19.5°, and about 11,700 above that temperature. In the case of *Zea* the values of the temperature characteristics above and below 19.5° are respectively about 13,100, and 21,050. The critical temperature at which the breaks occur is about 19.5° in both cases. A temperature characteristic of the order 16,400± calories is frequently encountered in connection with respiration (Crozier, 1924-25; Navez, 1928-29); in association with this, the magnitude 11,200± is of frequent occurrence. Temperature characteristics of magnitudes 13,000 (or a little less) and 21,000± in association, are not, however, un-

marked extent. It was considered quite unusual that such a change of rate should occur, for the work of various authors (*e.g.*, Warburg, 1926; Navez, 1928-29) on the respiration of both animals and plants indicates that the rate of respiration is constant with respect to time at least within short durations. That the effect is not due to lack of thermal adaptation is seen from the fact that except after the first few

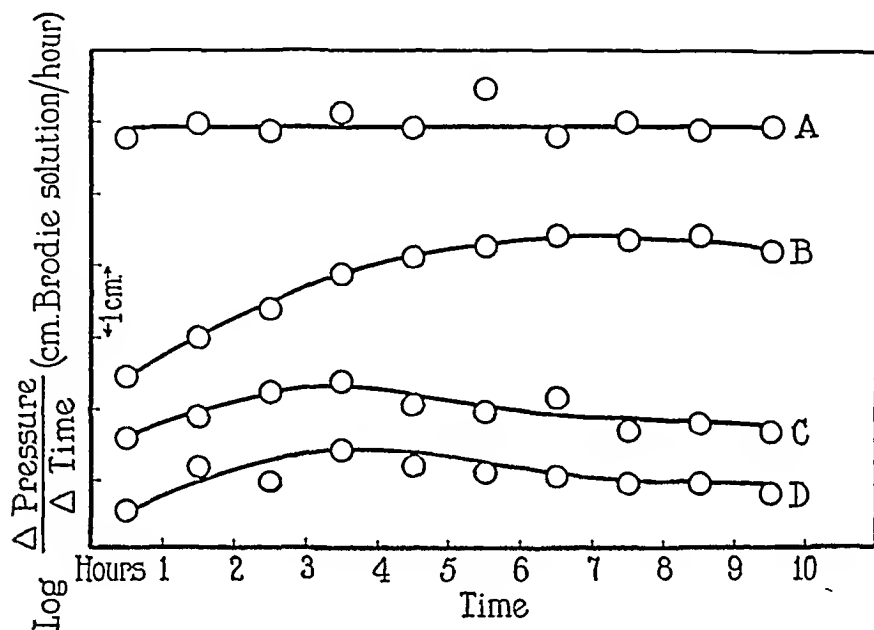


FIG. 6. The rate of oxygen consumption of *Lupinus albus* seeds which had been subjected to different conditions of germination, plotted against time, all at 18°. Curves *A* and *B* represent rates for seeds germinated on moist sawdust and transferred to the respiration vessels; *A* is 9 hours older than *B* at the time of transfer to the respirometer and the beginning of observations. *C* represents rates for seeds germinated over water, in a moist chamber, and then transferred to the respiration vessel. *D* represents rates for seeds germinated in the vessel over the alkali, without transfer. *B*, *C*, and *D* are similar in age, and are 9 hours younger than *A*.

readings the points on the graphs of Figs. 1 and 2 fall consistently on a straight line. Furthermore, a period of at least  $1\frac{1}{2}$  hour elapsed before the first readings in Fig. 5 were taken, and that length of time ought to have been enough for the small vessel and its contents to come to temperature equilibrium. The most probable reason for the occurrence of such an effect is that it is due to a change in the condition of the seed

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# THE FLOCCULATION OF GELATIN AT THE ISO-ELECTRIC POINT

By DANELLA STRAUP

*(From the Department of Chemistry and Food Research Institute, Stanford University, Stanford University)*

(Accepted for publication, February 26, 1931)

It is well known that such properties of gelatin solutions as turbidity and Tyndall effect intensity, which depend upon the aggregation of the gelatin, show a maximum at the isoelectric point. This relation has been studied quantitatively by Kraemer and Dexter (1927), who have shown that above 40°C. the light-scattering capacity of gelatin is very slight and is nearly independent of temperature and hydrogen ion concentration, but that below this temperature, particularly with calfskin gelatin, the scattering increases very sharply within a few tenths of a pH unit in the region of the isoelectric point. It was pointed out by these investigators that this behavior cannot be explained on the basis of the solubility of a homogeneous substance. By its separation into a "soluble" and an "insoluble" fraction by Schryver and Thimann (1927) and by Kunitz and Northrop (1928) it has furthermore been shown that gelatin itself is not a truly homogeneous substance.

The writer had observed that when gelatin solutions, at the isoelectric point and sufficiently free from electrolytes and other impurities, are cooled to temperatures below 40°C., aggregation occurs to such an extent that gelatin separates from solution as a flocculant precipitate. It was considered worth while to study this flocculation of gelatin in some detail, as it appeared to offer a method for investigating the nature of gelatin solutions and their possible heterogeneity. It also seemed to offer a possible method for the fractionation of gelatin.

The particular problems selected for this study were (1) the nature of the equilibrium between the flocculated and the dissolved gelatin, and (2) the properties of these gelatin flocculates and of the gelatin remaining in solution at equilibrium at various temperatures.





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## I. EXPERIMENTAL MATERIAL AND METHODS

Solutions were prepared by soaking gelatin in distilled water for 1 hour at room temperature, heating to 60°C. for 10 minutes, and filtering. They were then dialyzed in an electro-ultrafiltration apparatus of the type described by Bechhold (1925). Using direct current at 110 volts, electrodialysis was continued until the current passing through the solution had decreased to a constant low value, characteristic of the cell. Using a small dialyzer of 100 cc. capacity, it required less than 2 hours for the current to decrease to a constant value of 0.8 milliamperes. A larger dialyzer of 2 liters' capacity was less efficient, because of the relatively smaller electrode surface, and required 8 hours to reach a constant current of 8.0 milliamperes. Ultrafiltration membranes were prepared from a 10 per cent solution of collodion in glacial acetic acid; such membranes were found to give gelatin-free filtrates. The gelatin solutions were kept at 37°C. during dialysis to prevent the formation of a gel on the membranes and were stirred constantly by means of an electric stirrer.

The hydrogen ion concentration of those solutions which were not already at the isoelectric point changed rapidly during dialysis, finally reaching a value which remained constant during prolonged dialysis. On cooling such a dialyzed solution to temperatures below a range of 30–40°C., depending upon the concentration, gelatin separated out as a flocculant precipitate, which has been taken as the criterion that the solution is at its isoelectric point and free from electrolytes.

The pH value at which the solutions became isoelectric differed for various gelatins. Solutions of Coignet's Gold Label and of Coignet's Silver Label gelatin remained at a pH of 5.0 during dialysis, which is the isoelectric point for these gelatins. Solutions of Bacto-gelatin of the Digestive Ferments Company, originally at a pH of about 4.5 changed rapidly to a value within a range of pH 7.6 to 8.4, the isoelectric range for this gelatin, and then remained at this value during further dialysis. A pigskin gelatin (Ucopco gelatin of the United Chemical and Organic Products Company) became isoelectric at a pH of 6.8. A spray-dried powdered gelatin of commercial grade became isoelectric at a pH of 5.6.

It has been shown recently by Briefer (1929) that the isoelectric point of gelatin depends upon the previous chemical treatment. Kraemer and Dexter (1927) and Briefer and Cohen (1928) have obtained an isoelectric point at a pH of 5.0 for lime-treated calfskin gelatin and an isoelectric range around a pH of 8 for acid-treated pigskin gelatin. Sheppard and Houck (1930) have found a similar isoelectric point for calfskin gelatin and lime-treated pigskin gelatin, and isoelectric ranges of pH 7 to 8 and pH 6 to 7 for two samples of acid-treated pigskin gelatin. It would appear that the Ucopco gelatin and the Bacto-gelatin were of this latter character.

Before any comparison of the isoelectric flocculation of the various gelatins could be made, it was first necessary to study in detail the behavior of one particular gelatin in order to develop a suitable technique for the equilibrium study. Coig-

net's Gold Label gelatin was selected on account of its relative purity and has been used exclusively in the following experiments.

Solutions 2 and 3 were dialyzed in the 100 cc. dialyzer; Solutions 4 and 5 were dialyzed in the 2 liter dialyzer; a small amount of toluene was added before dialysis to prevent bacterial action during dialysis. Solutions 6, 7, and 10 were dialyzed in the 100 cc. dialyzer; no toluene was added to these solutions or to the following solutions. Solution 8 was dialyzed in the 2 liter dialyzer; portions of this solution were diluted to make Solutions 9 and 11. Solution 1 was dialyzed for a longer period of time (a total of 20 hours), and between periods of dialysis was heated four times to temperatures between 80°C. and 90°C. for periods of 10 minutes. All solutions were at a pH of 5.0 and had a specific conductivity of  $6 \times 10^{-6}$  to  $10 \times 10^{-6}$  reciprocal ohms.

When dialysis was completed each solution was pipetted into Pyrex test tubes, which were sealed in the blast lamp, and then heated to 80°C. for 10 minutes. Solutions treated in this manner showed no evidence of bacterial growth during the course of the experimental work and tubes now over 2 years old are still clear. This method of preventing bacterial action was found to be more efficient than the usual method in which antiseptics are employed and does not bring into question the effect upon flocculation of the addition of any foreign substance.

The chief objection which could be made to this method of preventing bacterial action is that irreversible changes are known to occur when gelatin solutions are heated. Sheppard and Houck (1930) have followed the rate of this change in concentrated gelatin solutions by measurement of viscosity. They found the change occurring at 40°C. at the isoelectric point to be extremely small and at 85°C. a 1 per cent change occurred in 10 minutes.

It was therefore necessary to conduct a preliminary investigation to determine a satisfactory time and temperature for the routine heating. It was soon found that heating solutions for 10 minutes at temperatures above 80°C. brought about an irreversible decrease in the amount of gelatin flocculating on subsequent cooling to 25°C., as well as a decrease in the rate at which this flocculation equilibrium is reached. It was also found that this effect increased rapidly with the temperature, apparently as a logarithmic function. At 80°C., no apparent effect on the flocculation was produced in 10 minutes while, as noted above, the bactericidal results seem entirely satisfactory; this time and temperature were accordingly adopted for all of the experimental work with the exception of Solution 1 which was used in Sections 1 and 2 following. The results of heating gelatin solutions at temperatures above 80°C. and for times longer than 10 minutes are of considerable interest and will be discussed subsequently in this paper.

After heating, the solutions were put into thermostats and, after flocculation began to occur, the gelatin, which remained in solution, was determined at regular intervals. A separate tube, not previously opened, was used for each determination, to avoid contamination with microorganisms.

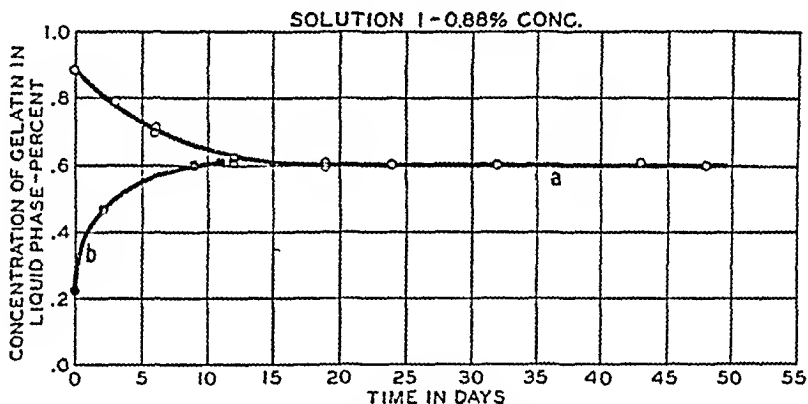
The concentration of gelatin in the solutions was determined by measurement

of the refractive index at 25°C. with a Zeiss immersion refractometer. A calibration, made by determining the refractive indices of solutions of known concentration, showed that the refractive index of a gelatin solution is a linear function of the concentration, as has been found also by Walpole (1913) and Sheppard and Houck (1930). Concentrations of gelatin could be determined by this means to within 0.01 per cent.

## II. EXPERIMENTAL

### 1. *The Establishment of Equilibrium in Flocculating Gelatin Solutions*

The flocculation of gelatin from solutions is temperature reversible. Experiments were made to determine the extent of flocculation at a



GRAPH 1. Rate of flocculation at 25°C. and redispersion at 25°C. of gelatin previously flocculated at lower temperature.

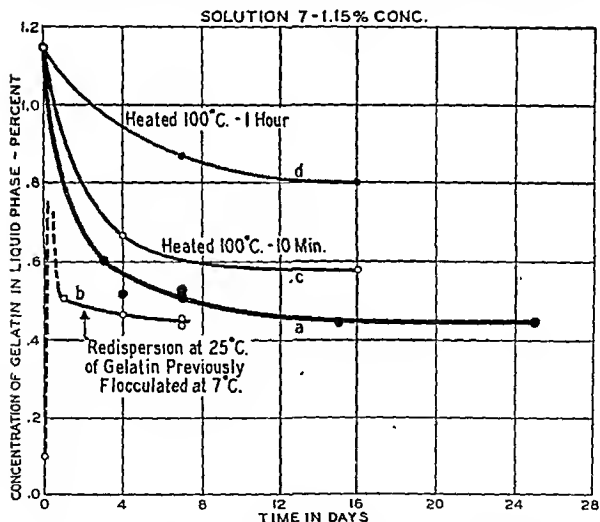
given temperature and whether the system would reach an equilibrium. Twelve tubes containing 0.88 per cent gelatin solution (Solution 1) were put in a thermostat at 25°C., immediately after their heating to 80°C., and were kept at this temperature for 45 days. The concentration of gelatin remaining in the liquid phase, determined at regular intervals, reached a constant value after 18 days, and at the end of 2 months this value had not changed (Graph 1, Curve *a*). Another set of tubes containing this same solution was brought to a temperature of 15°C. and flocculation allowed to occur. These tubes were then placed in the thermostat at 25°C. A portion of the gelatin flocculate redispersed (Graph 1, Curve *b*), and after 12 days at 25°C. the liquid

phase in these tubes contained the same concentration of gelatin as was obtained when flocculation occurred after direct cooling to 25°C.

Thus, at a given temperature, the same concentration of gelatin in the liquid phase is obtained whether the system is brought to that temperature from a lower temperature or from a higher temperature, and no further flocculation occurs after a certain length of time. This indicates that in such systems an apparent equilibrium exists between the gelatin flocculate and the gelatin remaining in solution at a given temperature.

## 2. Irreversible Change on Heating Gelatin Solutions

It has been mentioned that Solution 1 was heated several times to temperatures above 80°C. for periods of 10 minutes. This treatment



GRAPH 2. Rate of flocculation at 25°C. showing the effect of heating the solutions above 80°C.

produces irreversible changes; and, for this reason, the values at flocculation equilibrium for this solution cannot be compared with other solutions of the same concentration which were not heated above 80°C.

The magnitude of the effect of this irreversible change in decreasing the percentage of gelatin flocculation at any given temperature is shown in Graph 2. Curve *a* shows the rate at which flocculation occurred at 25°C. in Solution 7, of 1.15 per cent concentration, which had been heated only to 80°C. in the routine heating. Curve *c* shows the rate of flocculation at 25°C. for this same solution which was heated to 100°C. for 10 minutes, and Curve *d* for the same solution heated to 100°C. for 1 hour.

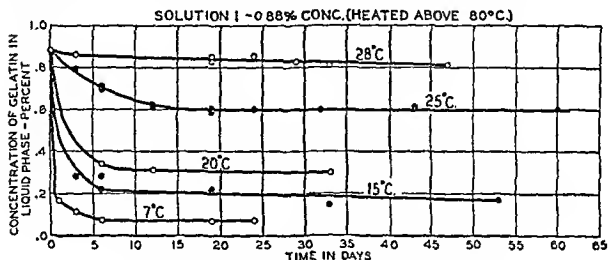
In solutions which have been heated at 100°C., even for several hours, there is, however, no change in the refractive index or hydrogen ion concentration. The fact that the effect upon flocculation of this heat treatment is not removed when the treated solutions are subsequently electrodyalyzed, indicates that this effect is not due to the presence of small amounts of ammonia, or other electrolytes, which might conceivably be produced by hydrolysis. In general, it may be said that there are no indications that the irreversible change on heating is due to any marked change in the chemical nature of the gelatin.

### *3. The Relation between Temperature and Flocculation Equilibrium*

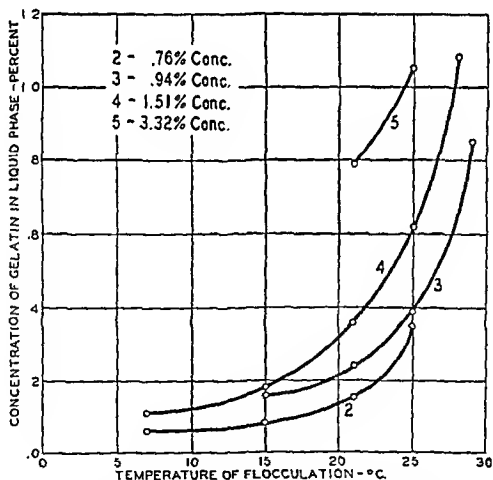
The flocculation equilibrium for Solution 1 was determined at a number of temperatures. It is seen from Graph 3 that flocculation was rapid at 7°C. and that after 6 days no further flocculation occurred. Flocculation equilibrium was approached more slowly at higher temperatures and at 28°C. had not been reached after 40 days.

The relation between the temperature of flocculation and the concentration of gelatin remaining in solution after flocculation has occurred is shown further for Solutions 2, 3, 4, and 5 in Graph 4. These curves resemble the temperature-gelation curve for gelatin systems which are not at the isoelectric point, or which contain sufficient electrolytes or other impurities to prevent flocculation. The aggregation process which results in flocculation of gelatin in the absence of electrolytes must be very similar to the aggregation process which results in gel formation when small amounts of electrolytes are present in the gelatin system. This aggregation is presumably the result of the loss

of thermal energy by the gelatin molecules. In the case of flocculation the coalescence of the molecules is more complete than in the formation of a gel.



GRAPH 3. Rate of flocculation at various temperatures.

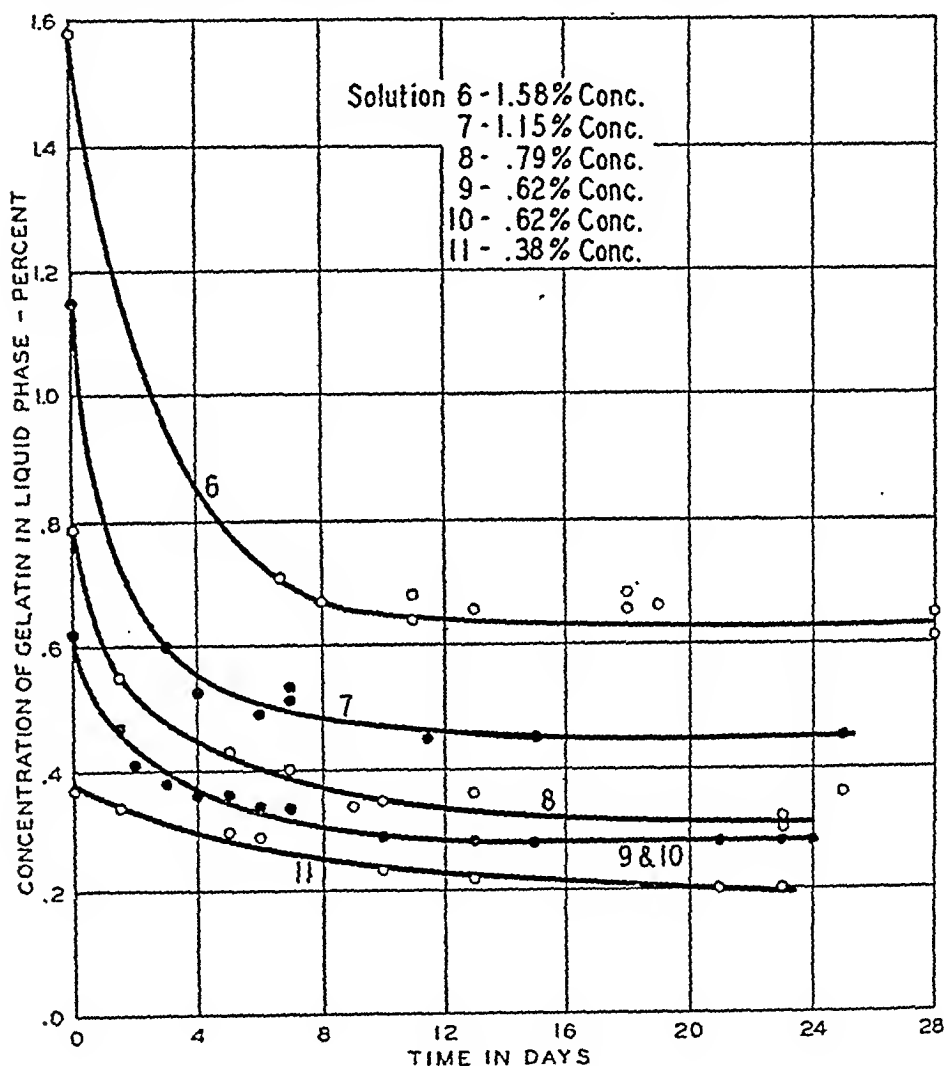


GRAPH 4. Temperature reversible flocculation of gelatin.



#### 4. Relation between Original Gelatin Concentration and Flocculation Equilibrium

The flocculation equilibrium at 25°C. was determined for Solutions 6 to 11, inclusive, of concentrations ranging from 1.58 per cent to 0.38 per cent



GRAPH 5. Rate of flocculation at 25°C. for solutions of various concentrations.

per cent. The large differences in the concentration of gelatin remaining in the liquid phase after flocculation in these solutions is shown in Graph 5. The concentration of gelatin remaining in solution is evi

dently dependent upon the concentration of the original solution, increasing nearly linearly with increasing amounts of gelatin in the original system. The slight departure from linearity may perhaps be accounted for by the fact that the rate at which equilibrium is established is lower for dilute gelatin solutions than for more concentrated solutions.

It has been shown above that a definite relation exists at any temperature between the flocculate and the gelatin remaining in solution. But this relation cannot be considered as an equilibrium condition of a single chemical substance of a definite solubility since the concentration of gelatin remaining in solution is dependent not only on the temperature but also on the amount of gelatin present in the system. A gelatin solution must, therefore, be considered either as a mixture of two or more chemical substances of different solubilities or as a mixture of particles similar in chemical nature but non-uniform in size, or some still more complex system must be assumed. In the following experiments an attempt is made to examine these several possibilities.

### *5. Flocculation from the Precipitate Fraction*

After flocculation had occurred in Solution 2 (0.76 per cent) at 25°C., the precipitate containing 53.8 per cent of the gelatin present was separated from the liquid phase. After washing several times with distilled water and centrifuging, the flocculate was dispersed in distilled water by warming and gave a solution of 1.33 per cent gelatin content. This solution was sealed in Pyrex tubes, heated to 80°C. for 10 minutes and put in the thermostat at 25°C. 83 per cent of the gelatin flocculated from this solution within 1 day.

In a preliminary experiment, a solution of 1.16 per cent concentration was allowed to flocculate at room temperature (about 18°C.). After washing, redispersing, and reflocculating twice, with the removal of the liquid phase each time, the precipitate was dispersed to form a solution of 1.03 per cent concentration. 90 per cent of the gelatin flocculated from this solution at room temperature within 1 day.

Thus, it is seen that flocculation of gelatin occurs in solutions of these precipitate fractions to a much greater extent and at a greater rate than in the original solution of the same concentration and at the same temperature. It is probable that the gelatin which remains in

solution after reflocculation in these precipitate fractions is chiefly gelatin which would normally have remained in solution at the original flocculation temperature but which was carried down mechanically. It would appear possible that, if the precipitate could be washed, redispersed, and reflocculated a sufficient number of times, a solution would be obtainable from which the gelatin would entirely flocculate at the original temperature of flocculation. It is intended that a thorough investigation be carried out on this point.

#### *6. The Redispersion of Low Temperature Flocculates*

An interesting phenomenon was observed when tubes in which flocculation equilibrium was established at 7°C., were brought to a temperature of 25°C. As soon as thermal equilibrium was reached at 25°C., the flocculate entirely disappeared to give an opalescent solution from which reflocculation quickly occurred. After several days, equilibrium was again reached and the liquid phase contained the same gelatin concentration as was obtained when the flocculation equilibrium was reached by lowering the temperature from 80°C. directly to 25°C. This is shown in Graph 2. Curve *a* shows the rate at which equilibrium was reached when the tubes were placed directly in the thermostat at 25°C. Curve *b* shows the changes occurring at 25°C. in the tubes in which flocculation equilibrium had been reached previously at 7°C.

This behavior in dispersion is best explained as a result of the intimate mixture in the 7°C. flocculate of particles which would be in solution at 25°C. with those which would be flocculated at that temperature, so that on heating to 25°C. the more dispersible particles exercise a disruptive force on the entire flocculate.

#### *7. Flocculation of Gelatin from the Liquid Fraction*

The liquid phase of gelatin Solution 3 was removed after flocculation had occurred at 25°C., and concentrated in the electro dialyzer from a gelatin content of 0.39 per cent to 1.17 per cent. This solution was then sealed in Pyrex tubes and heated to 80°C. as usual. At 25°C., this solution remained clear and fluid for over 30 days, no flocculation occurring. When the temperature of this solution was lowered to 21°C., 29 per cent of the gelatin had flocculated in 3 days and 48 per

cent in 30 days. At 15°C., 65 per cent flocculated in 2 days and 76 per cent in 30 days.

Similarly a solution was prepared by removing the liquid phase from Solution 2 after flocculation had taken place at 25°C. and concentrating from a gelatin content of 0.34 per cent to 1.22 per cent. No flocculation occurred as long as the temperature of this solution was kept at 25°C. At 15°C., 68 per cent of the gelatin precipitated in 3 days; and at 7°C., 83 per cent in the same time.

A solution containing 0.81 per cent gelatin was flocculated at 21°C., 75.3 per cent of the total amount of gelatin precipitating. The liquid phase of 0.20 per cent gelatin content, so obtained, was concentrated to 1.0 per cent. No flocculation occurred at 21°C. At 15°C., 40.8 per cent of the gelatin flocculated in 26 days leaving a liquid phase of 0.68 per cent gelatin concentration.

The flocculation of gelatin from liquid fractions resembles the flocculation from the original solution, but occurs only over a temperature range below the original flocculation temperature. This is further strikingly shown by the behavior of the liquid fraction obtained from a very concentrated gelatin solution (about 8 per cent) at 25°C. This liquid, containing 2.39 per cent gelatin, remained clear and fluid at 25°C. but at lower temperatures flocculation occurred.

The behavior of solutions of these liquid and precipitate fractions is difficult to explain by assuming that the non-uniformity of gelatin is due to the presence of only two constituents or of even a small number of constituents, whether they differ chemically or physically. There is evidence to indicate that this non-uniformity exists in the liquid fraction as well as in the original solution. It has been shown above that a liquid phase obtained at any temperature can be concentrated to one-tenth of its volume without flocculation occurring as long as the temperature is not lowered below that of the original flocculation. It must be assumed, then, that the constituents of the original precipitate are entirely insoluble in the liquid phase at that temperature. Otherwise, a further precipitation of these constituents would occur when the solution was concentrated. In the experiments with Solutions 2 and 3 above, the concentrated liquid fraction, obtained by flocculation at 25°C., gave no precipitate at 25°C. but, when cooled to 21°C., 15°C., and 7°C., an increasingly large partial flocculation occurred. It

is evident that these partial flocculations cannot be due to the separation of a residual portion of the constituent or constituents represented in the original flocculate, but must indicate a heterogeneity in the 25°C. liquid fraction. Confirming evidence of this heterogeneity of liquid fractions has also been obtained from the observation that, in them also, flocculation is a function of both temperature and concentration.

The same reasoning may be applied to any fraction. Since it appears that the number of fractions obtainable depends simply upon the number of temperatures at which flocculations and separations are made, it would seem that the number of species of gelatin constituents present must be assumed to be large. These gelatin constituents may represent molecules differing in chemical structure or they may be the result of different degrees of disruption of the collagen macromolecule, differing in size rather than in chemical constitution. As noted above, there is evidence favoring the latter assumption. In either case it would be expected that, at definite temperatures, particular constituents would lose sufficient thermal energy to form loose aggregates and flocculate from solution. On raising the temperature, these aggregates would be broken up to give a solution of the original constituents. The change occurring when gelatin solutions are heated to higher temperatures may be regarded as a further irreversible breaking up of the various gelatin constituents. According to this conception, when flocculation has occurred in gelatin solutions at 25°C., all gelatin constituents which tend to precipitate at this temperature have been removed from the liquid phase and no further flocculation should occur on concentration. On lowering the temperature, however, another group of constituents tends to precipitate.

#### *8. The Salting-Out of the Flocculate Fraction and of the Liquid Fraction*

Further evidence concerning the heterogeneity of gelatin is given by the different behavior of solutions of the liquid fraction and of the precipitate fraction when these solutions are salted out with ammonium sulfate.

Varying amounts of a saturated solution of ammonium sulfate were added to 10 cc. portions of a gelatin solution containing only the precipitate fraction, and to 10 cc. portions of a solution containing only the

liquid fraction, obtained by flocculation at 25°C. Both solutions were at 25°C. and of the same concentration, 0.90 per cent. In the case of the precipitate fraction, the solution became turbid when 5.6 per cent saturated with ammonium sulfate. The turbidity increased gradually on the addition of more ammonium sulfate. At 9 per cent of saturation, a small amount of precipitate had formed. The amount of this precipitate increased gradually with further addition of ammonium sulfate, until the solution was 16 per cent saturated, when the precipitate flocculated out completely leaving a clear solution. In the case of the liquid fraction, no turbidity appeared until the solution was 14 per cent saturated, when just a faint opalescence occurred. At 15 per cent of saturation, a heavy precipitation occurred.

The behavior of these fractions of gelatin with respect to precipitation by ammonium sulfate can also be explained by the non-uniformity of the gelatin constituents. Thus, solutions of the precipitate fraction, obtained at 25°C., contain those classes of constituents which are most easily precipitated. There are present in this fraction only a small number of constituents which are precipitated by a low concentration (5.7 per cent of saturation) of ammonium sulfate. But there also are present other classes of constituents which can be precipitated by higher concentrations of ammonium sulfate. Hence, on increasing the concentration of ammonium sulfate, the amount of precipitation increases gradually. In the case of the liquid fraction obtained at 25°C., no precipitation occurs for low concentrations of ammonium sulfate since all classes of constituents which can be precipitated by concentrations less than 15 per cent of saturation have already been removed from this fraction. From the large and sudden precipitation which occurs in this fraction at 15 per cent of saturation, it appears that gelatin contains a relatively large proportion of constituents of this group. The number of constituents which require more than 15 per cent of saturation to cause precipitation is relatively small.

At first sight it would seem that the data from the precipitate fraction are somewhat in conflict with those from the liquid fraction; that is, complete precipitation in the precipitate fraction occurred at 16 per cent of saturation while the heavy precipitation in the liquid fraction took place when only 15 per cent saturated with ammonium sulfate. This apparent overlap in the characteristics of the two solutions

can easily be explained. The degree of ammonium sulfate saturations, as stated, is only an indication of the amount of sulfate added and not of the actual concentration present. It is probable that a certain amount of ammonium sulfate is removed from the solution by the flocculate and that, in the case of the precipitate fraction, the nominal 16 per cent saturation at which the heavy precipitation took place was actually somewhat less. The seemingly complete salting out of the liquid fraction at 15 per cent of saturation indicates a carrying down of the less precipitable species during the heavy precipitation which occurs when the boundary between the original precipitate and liquid fraction is passed. This is apparently the same phenomenon that was observed in the case of rapid flocculations at low temperatures.

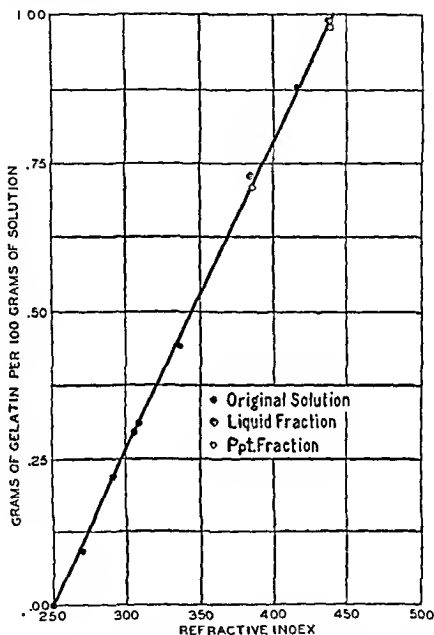
The same marked difference in the behavior of the precipitated fraction and of the liquid fraction toward salting out with ammonium sulfate was also observed at 60°C. This would seem to show that the characteristic differences between the gelatin constituents persist at temperatures above the isoelectric flocculation range. This is also indicated, of course, by the unchanging behavior of precipitate fractions when repeatedly redispersed and flocculated.

#### *9. Refractive Indices of the Liquid and of the Precipitate Fractions*

It is shown in Graph 6 that the refractive indices for solutions of the liquid fraction and of the precipitate fraction are, within the experimental error, the same linear function of the concentration as for the original solution. From both the liquid and precipitate fractions of Solution 1, flocculated at 25°C., solutions of two different concentrations were prepared. The refractive indices of these two sets of solutions were determined and those of the same concentration from either fraction were found to be identical within the experimental error. This indicates that there can be no very great chemical difference between the gelatin constituents of these fractions. Since refractive index is dependent upon the basic molecular structure and not upon the size of the constituents, the assumption that gelatin consists of a large number of constituents which differ only in the degree of association or orientation offers the best explanation for the observed facts.

If this explanation is correct, a marked difference should exist be-

tween the liquid and the precipitate fractions with respect to some physical characteristic, such as viscosity, which is dependent upon the degree of association or orientation.



GRAPH 6. Relation between refractive index and concentration. The figures on the abscissa are abbreviations for 1.33250 etc.

#### 10. Viscosity of the Liquid and of the Precipitate Fractions

The previously discussed irreversible change, occurring on heating, causes a decrease in the viscosity of gelatin solutions and a decrease in the amount of gelatin which flocculates from solution at any given temperature. It is to be expected, therefore, that the liquid fraction



would have a lesser viscosity than the precipitate fraction. This was found to be the case. The viscosities of the original solution and of solutions containing the precipitate fraction flocculated at 25°C. and of the solutions containing the liquid fraction after flocculation at 25°C. were determined. Viscosities were measured in an Ostwald viscosimeter at 40°C., at which temperature it has been shown by Hatschek and Jane (1926) and others that the rate of flow through a capillary is a measure of true viscosity. It was found that the viscosity of the original solution is intermediate between the viscosity values of the precipitate fraction and of the liquid fraction and that flocculation can effect a separation into categories exhibiting a marked difference in a physical characteristic.

### III. CONCLUSIONS

The results of this investigation show that a gelatin solution consists of a considerable number of constituents. At a particular temperature, certain gelatin constituents tend to aggregate and to flocculate from solution. When these particular gelatin constituents have completely flocculated, no further change occurs in the system and an apparent equilibrium exists. This is not a dynamic equilibrium between the gelatin flocculate as a whole and the gelatin remaining in the solution but a steady state determined for that system by the temperature.

It is also shown that gelatin can be separated into fractions in which the gelatin constituents are more nearly uniform and tend to flocculate over a much narrower temperature range. It should be possible to obtain a number of fractions in which all of the gelatin would flocculate at a definite temperature. The aggregation of the various gelatin constituents is presumably due to loss of thermal energy, and the temperature at which this occurs must be some function of the mass of the constituent. It is natural to assume, then, that the constituents which flocculate at a given temperature are larger than those which remain in solution at that temperature. Recently, Krishnamurti and Svedberg (1930) have obtained evidence with the ultracentrifuge that the constituents of a gelatin solution are heterogeneous as to mass, even at a pH value at which there is no tendency toward aggregation.

There is much reason to suppose that the gelatin constituents do not differ very greatly chemically since different fractions have the same refractive index and the same isoelectric point.

The data as a whole are best explained by considering the gelatin constituents to be different degrees of association of the same or very similar molecular structural units. This is in agreement with Shepard and Houck (1930), who consider that "the molecules of gelatin are fundamentally identical with those of collagen, the difference being only in the degree of association and orientation". Meyer and Mark (1928) have interpreted the x-ray data obtained from collagen as indicating that the micelles of the collagen fiber are built up of main valency chains of anhydro-amino acids. It may be supposed that during peptization of these fibers, the amino acid chains become separated, disorientated, and partially broken up, so producing the heterogeneous system which we know as gelatin. It is evident that the manner in which this breaking-up proceeds depends upon the chemical treatment previous to the peptization process and the gelatin produced from lime-treated collagen would be expected to differ from that from acid-treated collagen.

From the results herein reported it seems evident that the technique of isoelectric flocculation of electrolyte-free gelatin offers a profitable method for the study of gelatin and an extended investigation along these lines should yield much valuable information concerning the nature of gelatin. It is possible that this method may also be extended to other hydrophilic colloids.

In conclusion, the writer wishes to express her sincere appreciation of the sympathetic assistance of Dr. C. L. Alsberg, under whose guidance this work was carried out.

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# THE VAPOUR PRESSURES OF AQUEOUS SOLUTIONS WITH SPECIAL REFERENCE TO THE PROBLEM OF THE STATE OF WATER IN BIOLOGICAL FLUIDS

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## I

### INTRODUCTION

The study of the physical properties of solutions of a number of organic and inorganic substances in water has led observers, from time to time, to assume the existence of combinations between the ions or molecules of the solute with the solvent. Thus the deviation of the colligative properties (freezing point depression, osmotic pressure, etc.) of certain solutions from the theoretically calculated values has been explained as due to such hydrate formation (14), although alternative hypotheses have always been possible, e.g., that the solute produces the observed deviations by shifting the equilibrium existing normally between the various states of aggregation of water. Transport experiments have likewise led to the view (22) that ions are hydrated but the results thus obtained are open to criticism (30) as are also the results obtained by the combination of transport numbers with Stokes's law for the motion of a sphere in a viscous medium. The results obtained by such methods are, moreover, in marked disagreement with one another. Thus for the chloride ion, colligative properties give no indication of hydration; transport experiments (assuming the hydrogen ion to hold one molecule of water) indicate the chloride ion to be combined with four molecules of water; application of Stokes's law (assuming the hydrogen ion not to be hydrated) gives the hydration number of the chloride ion as twenty; while application of the Stokes-Einstein formula for the motion of particles in a

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viscous medium for determining the radii of ions has led Lorenz (17) to deny any hydration by the chloride ion. Assuming the existence of water of hydration, our knowledge of the extent to which such water differs in its behaviour from the remainder of the solvent is quite fragmentary.

The problem of the state of water in colloidal solutions and biological fluids also has been a matter of controversy in recent years. It has often been argued that much of the water present in colloidal solutions is bound to the solute and hence differs in its properties from the remainder of the water which acts as a solvent for the *solute-water* combination. The question whether such combination actually occurs and, if so, how the water in such systems differs from the water in simple solutions is of fundamental importance. The existence of "bound" water differing in its reactions from that of the solvent might permit reactions impossible in simpler solutions. Indeed such possibilities have been advanced to explain certain biological phenomena, but the proof for the existence of such "bound" water has been based on rather dubious data. The freezing methods of Jensen and Fischer (13), Rubner (24), and Thoenes (32) are open to a number of objections. Moran (19, 20), by the use of a dilatometric method, has recently been unable to confirm the results so obtained. Another method which has been utilized for determining the state of water in solutions depends on the determination of some colligative property (freezing point or vapour pressure) of a substance in the solution under investigation and comparison of the result with that obtained when the same substance is dissolved in pure water. Gortner (6) and his collaborators, utilizing sucrose and freezing-point depression measurements, adduced evidence which they believed to indicate the existence of "bound" water in colloidal solutions. A. V. Hill (11), utilizing his thermal method for determining vapour pressure, demonstrated the absence of any appreciable amount of "bound" water in blood, muscle, etc. He added a number of substances (NaCl, KCl, sucrose, etc.) to the solutions investigated. Certain organic substances—urea, creatine, lactic acid, and succinic acid—gave inconsistent results which were attributed to adsorption or other possible interfering reactions.

The term "bound" water has been used by most authors in a vague sense, but Hill (11) has clearly defined the term to mean water which

is not capable of dissolving in a normal manner (with normal depression of vapour pressure) substances added to it. It was in the sense of this definition that the present investigation was undertaken to determine the state of water in solutions and the extent to which we may apply the colligative method described above for differentiating "free" and "bound" water. This method assumes that the colligative properties of a solution containing several solutes is an additive function of the properties of the individual constituents. Our knowledge of such solutions, however, is very limited, although of greatest importance in biological fluids which contain usually many solutes. The present study, therefore, was made to include measurements of the vapour pressure lowering of solutions containing several solutes as compared to the vapour pressure lowerings produced by the individual constituents.

## II

### *Experimental Methods*

The vapour pressure measurements recorded in the present paper were made by the thermal method devised by Hill (10) as described in the papers of Hill (10, 11) and Margaria (18). The determinations were carried out at 20.3°C. in a water bath maintained constant to  $\pm 0.001^\circ$ .

It was necessary in the present work to determine the vapour pressure depressions of dilute aqueous solutions (0.01 to 0.1 molar) for which previous methods (the static or dynamic) are quite unsuitable. Hill's thermal method is accurate at such low concentrations and, in fact, yields results which are of greater absolute accuracy than for more concentrated solutions. The study of dilute solutions requires the use of highly sensitive galvanometer and thermopiles. The galvanometer used in the present work was the moving magnet type described by Downing (3) but modified by making the system sufficiently astatic to render shielding unnecessary (3). The instrument had an internal resistance of 50 ohms, with the coils in series, and its sensitivity was altered by manipulating an external magnet. The sensitivity was fixed so that the most dilute solutions used (0.01 molar) gave a deflection (against water) of about 100 mm. on a scale 2 metres distant from the galvanometer.

The sensitivity of the apparatus may also be increased by decreasing the distance between the walls of the tube (in which the thermopile is inserted) and the thermopile. Thus, reducing this distance to 1 cm. by the use of stationary parallel plates on which the solution, normally placed on the walls of the tube, was put, increased the sensitivity of a given thermopile 60 per cent. This phenomenon is due to the existence of a gradient of vapour pressure between the neutral solution

on the wall and the solution on the thermopiles. Proximity increases this gradient and produces a more active exchange by evaporation or condensation at the thermopiles, and hence the development of a greater temperature difference between their two faces.

From the effect on the sensitivity of the instrument of the distance between thermopile and wall it is clear that it is essential for high accuracy to maintain the spatial relationship (between thermopile and wall) constant in successive experiments. This is best done by the use of a metal tube with a fixed arrangement for its attachment. The use of a metal tube, moreover, hastens the attainment of equilibrium particularly if it is short and closely fitted to the thermopile. By reducing the size of the air space in the tube in which the thermopile is placed, one not only increases the sensitivity of the instrument but also materially hastens the attainment of equilibrium. The smaller the air space, the less tendency is there for the development of convection currents due to the unavoidable temperature differences existing in such spaces and the more rapidly is a dynamic equilibrium established between the three solutions present in the chamber.

In the present work the method of reversing the solutions on the two faces of the thermopile, to avoid errors due to asymmetry of the instruments (18), was employed. Such reversal, however, is unnecessary if a series of determinations is made in which the solution used as the reference substance and the one placed on the wall of the tube are not altered in composition during the course of the determinations. If, for example, a solution whose vapour pressure is  $p_a$  is to be studied, we need only place it on one face of the thermopile with another solution or water (whose vapour pressure we may call  $p_b$ ) on the opposite face and compare the reading so obtained with the reading from a similar arrangement in which a solution of known vapour pressure,  $p_c$ , replaces  $p_a$ . The excess temperature of the first face of the thermopile will be  $K_1(p_d - p_a)$ , where  $p_d$  is the vapour pressure of the solution on the wall of the tube. The excess temperature on the other face will equal  $K_2(p_d - p_b)$  and the final temperature read will equal  $K_1(p_d - p_a) - K_2(p_d - p_b)$ . In the case of our second solution likewise the final temperature read will equal  $K_1(p_d - p_c) - K_2(p_d - p_b)$ . The difference between the two sets of readings is  $K_1(p_c - p_a)$ . The last expression shows the difference between the observed readings to be directly proportional to the vapour pressure difference of the two solutions and to be unaffected by the asymmetry of the instrument.

The avoidance of reversal of the solutions on the thermopile is useful in cases where the fluid available is insufficient for duplicate experiments, or where a lesser degree of accuracy is acceptable in lieu of the extra labour involved in double determinations. Where the highest possible accuracy is required, however, it is desirable to reverse the solutions in successive determinations thus avoiding the possibility of an interference by extraneous electromotive forces in the external circuit. Such extraneous currents, although constant, will cause appreciable errors, except in cases where  $p_c$  and  $p_a$  (in the above example) are approximately equal.

The probable errors of the results for the dilute solutions recorded in the present

paper are approximately 0.5 per cent of the recorded reading. In the case of the more concentrated solutions studied, the probable error is about 1 per cent. Reversed readings were made with four thermopiles, the averages being recorded in the tables. Two sets of such readings (sixteen readings in all) were made for the dilute solutions of KCl and NaCl and a single set of reversed readings (eight in all) for the other results quoted. The probable error was found to decrease but slightly when more than four sets of determinations were made.

All the results given in the tables of the present paper are based on actual determinations which have been interpolated, where necessary, to the nearest 0.01 of a mole for convenience of recording.

TABLE I

*The Vapour Pressure Depressions of Dilute Aqueous Sodium Chloride Solutions at 20.3°C.*

| Moles of NaCl per 1000 gm. of H <sub>2</sub> O..            | 0.03        | 0.04        | 0.05        | 0.06        | 0.07        | 0.08        | 0.09        | 0.1         |
|---|-------------|-------------|-------------|-------------|-------------|-------------|-------------|-------------|
| Molal relative vapour pressure lowering* $\times 10^{-2}$ . | 3.42        | 3.39        | 3.37        | 3.34        | 3.32        | 3.31        | 3.30        | 3.29        |
| Probable error.....   | $\pm 0.018$ | $\pm 0.010$ | $\pm 0.012$ | $\pm 0.014$ | $\pm 0.010$ | $\pm 0.007$ | $\pm 0.008$ | $\pm 0.007$ |

\* The molal relative vapour pressure lowering is defined by the expression  $\frac{P_s - P}{MP_s}$ , in which  $P_s$  is the vapour pressure of the solvent;  $P$  that of the solution, and  $M$  is the number of gram formula weights of solute per 1000 gm. of solvent.

### III

#### *The Vapour Pressures of Dilute Aqueous Sodium and Potassium Chloride Solutions*

In determinations of vapour pressure by Hill's method comparison is made between the solution to be investigated and one whose vapour pressure is known (or water in the case of very dilute solutions). In the present work it was required to know the vapour pressure of very dilute solutions (less than 0.1 molar) in which range data are not available due to the errors in the usual methods for determining the vapour pressures of solutions of such low concentrations (8). Determinations, therefore, were carried out with dilute NaCl and KCl solutions, the results of which may be used as standards for vapour



pressure studies involving other dilute solutions. The data for NaCl, given in Table I, are based on the value of  $3.29 \times 10^{-2}$  as the molal relative lowering for a 0.1 molal solution as found by Norris (12). The data for KCl, given in Table II, are based on those of Table I. The value,  $3.35 \times 10^{-2}$ , for the 0.05 molal KCl solution is in good agreement with that of Frazer, Lovelace, and Sease (12) for this concentration,  $3.36 \times 10^{-2}$ , considering the probable error of the present results and those obtained by the static method for very dilute solutions (8, 16).

The molar vapour pressure lowerings of dilute sucrose solutions were found to be  $1.78 \times 10^{-2}$  for a 0.1 molal solution and  $1.77 \times 10^{-2}$

TABLE II

*The Vapour Pressure Depressions of Dilute Aqueous Potassium Chloride Solutions at 20.3°C.*

| Moles of KCl per<br>1000 gm. of H <sub>2</sub> O..                | 0.03        | 0.04        | 0.05        | 0.06        | 0.07        | 0.08        | 0.09        | 0.1         |
|---|-------------|-------------|-------------|-------------|-------------|-------------|-------------|-------------|
| Molal relative vapour<br>pressure lowering $\times 10^{-2}$ ..... | 3.41        | 3.38        | 3.35        | 3.33        | 3.31        | 3.30        | 3.29        | 3.28        |
| Probable error.....   | $\pm 0.015$ | $\pm 0.014$ | $\pm 0.012$ | $\pm 0.010$ | $\pm 0.013$ | $\pm 0.010$ | $\pm 0.008$ | $\pm 0.006$ |

for a 0.05 molal solution—values which are in good agreement with those of Beard (12) for the 0.1 molal solution at which concentration only, data are available.

#### IV

##### *The Vapour Pressures of Aqueous Solutions of Urea and Other Organic Substances*

As stated above, urea when added to blood was found by Hill (11) to give anomalous results for its vapour pressure lowering as compared to its solution in 1 per cent NaCl. The wide distribution of urea in biological materials renders important a knowledge of its colligative properties. The vapour pressures of aqueous urea solutions as found in the present work are given in Table III.

It will be observed that the molar relative vapour pressure lowering of aqueous urea solutions is constant (within the limits of the experimental error) over the range of 0.05 to 0.5 molar. The average molar relative lowering for these dilute solutions, however, is  $1.64 \times 10^{-2}$  which is appreciably lower than the theoretical value calculated on the assumption that urea exists in aqueous solution as a simple molecule. It would appear that urea is either associated in aqueous solution or affects the activity of the solvent in such a way as to lead to the abnormally low molal vapour pressure lowerings observed. Jones (14) also noted the abnormally low freezing point depressions of urea solutions which he attributed to association. The *freezing point depression-concentration* curve for urea as found by Jones differs from that for the other associated substances, which he studied, in the compara-

TABLE III

*The Vapour Pressure Depressions of Dilute Aqueous Urea Solutions at 20.3°C.*

| Moles of urea per 1000 gm.<br>of H <sub>2</sub> O.....         | 0.05        | 0.10        | 0.15        | 0.2         | 0.3         | 0.4         | 0.5         |
|--|-------------|-------------|-------------|-------------|-------------|-------------|-------------|
| Molal relative vapour pressure lowering $\times 10^{-2}$ ..... | 1.66        | 1.64        | 1.65        | 1.63        | 1.63        | 1.65        | 1.64        |
| Probable error.....  | $\pm 0.017$ | $\pm 0.013$ | $\pm 0.012$ | $\pm 0.010$ | $\pm 0.008$ | $\pm 0.007$ | $\pm 0.007$ |

tive constancy of the molal freezing point for dilute solutions. This observation is in accord with the constancy of the molal vapour pressure lowerings observed in the present work. Frankel (5) has recently observed the constancy of the molal refractive index of urea solutions. Such constancy, although an indication of an apparently constant molecular weight, is no criterion necessarily of the condition of the dissolved substance as is evidenced by the results of Jones and of the present study.

Dieterici (12) and Perman and Price (12) have also found abnormally low vapour pressures of urea solutions at 0° and 70°, respectively. Dieterici's results lead to the same conclusion regarding the abnormality of urea solutions at 0° as those deduced by Jones (14) from freezing point data as opposed to the normal freezing point lowering

observed by Flügel (4). Hendricks (9) has also shown that crystalline urea consists of molecules having the formula  $(\text{CON}_2\text{H}_4)_2$ .

Abnormality of the colligative properties of aqueous solutions of organic compounds is much more common than is ordinarily supposed. A normal colligative property appears to be the exception rather than the rule among organic compounds. Such abnormalities have been observed by Jones (14) for the depression of the freezing points of succinic, oxalic, citric and acetic acids, ethyl alcohol, acetone, etc.; by Frankel (5) for the refractive indices of aniline, glycol, phenol, formic and propionic acids, malonic ester, etc.; by Grollman (7) for the directly measured osmotic pressures of phenol; and by others, using various methods of experimentation, for a number of substances (12).

Comparison of the data of the present paper with those of Dieterici and Perman and Price, quoted above, would indicate that the association of urea (if such be assumed to occur) increases with increasing temperature. At the temperature of the mammalian body, one would therefore expect a greater abnormality of the colligative properties of urea than that observed at lower temperatures. This increase in the degree of association with increasing temperature is of frequent occurrence in the case of organic substances (12), although the reverse may be true in the case of some (*e.g.* phenol (7)). Consequently it is unjustifiable to assume a normal molecular weight in calculating the colligative properties of an organic substance, as is usually done, despite the fact that the substance manifests a normal depression of the freezing point.

In view of the abnormal behaviour of aqueous urea solutions described above, the effect of adding to such solutions other substances was investigated, for it is in such complex mixtures that urea occurs biologically. The method used in investigating these mixtures and those to be subsequently described was as follows. The solute (urea in the present case) was added to an aqueous solution of known composition. The resultant mixture was placed on one side of the thermopile while a portion of the original solution was placed on the opposite face. The resulting reading measured the vapour pressure lowering produced by adding urea to the solution in question. A second experiment was then performed in which the vapour pressure of an aqueous urea solution was determined. The ratio of the readings

obtained in the two experiments above described is equal to the ratio of the vapour pressure lowering produced by urea when added to the solution investigated to its lowering in aqueous solution. For example, the reading of a solution which was 0.1008 molar with respect to urea and 0.0272 molar with respect to NaCl was 548 (deflection in mm. on the galvanometric scale) when the solution was placed on one side of the thermopile with a 0.0272 molar NaCl solution on the other side. A second solution of urea (0.1012 molar in composition) when

TABLE IV

*The Vapour Pressure Lowerings Observed on Adding Urea to Various Solutions as Compared to Those Observed on Adding Urea in Equal Concentrations to Pure Water*

| Solution                                     | pH            | Molality of urea in final solution  | Ratio of the vapor pressure depression caused by the addition of urea to the solution as compared to that produced when dissolved in pure water |
|--|---------------|-------------------------------------|---|
|  |               | moles per 1000 gm. H <sub>2</sub> O |   |
| 1 per cent NaCl solution.....                | 7.0           | 0.12                                | 1.00  |
| Frog Ringer's.....                           | 7.0           | 0.10                                | 1.00  |
| 1 per cent gelatin.....                      | 7.0           | 0.10                                | 1.00  |
| Sea water.....                               | 8.2           | 0.12                                | 1.04  |
| 0.2 M Na <sub>2</sub> HPO <sub>4</sub> ..... | 8.8           | 0.12                                | 1.07  |
| 0.13 M NaHCO <sub>3</sub> .....              | 8.6           | 0.12                                | 1.03  |
| Very dilute lactic acid.....                 | less than 6.0 | 0.12                                | 1.07  |
| Very dilute NaHCO <sub>3</sub> .....         | 7.4           | 0.12                                | 1.09  |
| 2 per cent glycerin.....                     | 7.0           | 0.12                                | 0.96  |
| 1.0 M sucrose.....                           | —             | 0.15                                | 0.90  |
| “.....                                       | —             | 0.3                                 | 0.80  |
| 1 per cent gelatin.....                      | 5.0           | 0.12                                | 0.95  |

opposed by water on the second face of the thermopile gave a reading of 552. The ratio of the vapour pressure lowering of urea in a 0.0272 molar NaCl solution to its lowering in water is therefore

$$\frac{548}{552} \times \frac{0.1012}{0.1008} = 1.00$$

In other words, for the mixed solution of NaCl and urea in question, the vapour pressure lowering is an additive function of the lowering of the individual constituents.

Table IV gives the vapour pressure lowerings produced by urea when added to a number of solutions as compared to the vapour pressure depression of its aqueous solution. In certain cases, *e.g.* 1 per cent NaCl, 1 per cent gelatin (at pH 7.0) and Ringer's solution, urea behaves as anticipated, *i.e.*, it produces the same depression of the vapour pressure of these solutions as it does when added in equal concentration to pure water. In many solutions, however, its effect on the vapour pressure is different from what would be anticipated from its behaviour in pure water. This anomalous behaviour may be assumed to result from a shift in the equilibrium existing in aqueous

TABLE V

*The Vapour Pressure Lowerings of Various Aqueous Solutions Containing Creatine, Lactic Acid, and Succinic Acid*

| Solvent                          | Solute        | Molality of solute                       | Molal relative vapour pressure lowering $\times 10^{-2}$ | Ratio of molal vapor pressure lowering observed to that in H <sub>2</sub> O |
|----------------------------------|---------------|--|--|---|
|                                  |               | <i>moles per 1000 gm. H<sub>2</sub>O</i> |  |   |
| Water.....                       | Creatine      | 0.024                                    | 1.81   | —   |
| " .....                          | Lactic acid   | 0.034                                    | 2.03   | —   |
| " .....                          | Succinic acid | 0.1                                      | 1.91   | —   |
| 1 per cent gelatin (pH = 7.0) .. | Succinic acid | 0.1                                      | —  | 0.90  |
| " ..                             | Lactic acid   | 0.035                                    | —  | 0.80  |
| M sucrose.....                   | Lactic acid   | 0.041                                    | —  | 1.18  |
| " .....                          | Succinic acid | 0.1                                      | —  | 1.20  |

urea solutions by the addition of various substances to the solution. Acid and alkali apparently favour dissociation thus giving values greater than unity in the last column of Table IV. The maximum values obtained (1.09) correspond to the theoretically calculated values for urea. Substances such as sucrose or gelatin (pH 5.0), giving values (in the last column of Table IV) which are less than unity, may be considered as favouring association of the urea molecules or producing some other change leading to an increased vapour pressure of the solution. In blood also, as shown by Hill (11), urea shows an abnormally low ratio.

Other organic substances—creatine, lactic acid, and succinic acid—were also found by Hill (11) to give anomalous results when added to blood. In view of the abnormal behaviour of the colligative properties of these substances when dissolved in water, one might anticipate a similarity in their behaviour in complex solutions (*i.e.* those containing several solutes) to that found for urea. The results obtained with these substances in aqueous solution and in solutions of 1 per cent gelatin and molar sucrose are given in Table V. The marked effect of the latter substances on the apparent vapour pressure lowerings of lactic acid and succinic acid is shown in the last column of Table V. The same conclusions as were discussed above regarding urea, may be drawn concerning the behaviour of these substances.

## V

*The Effect of Added Sucrose on the Vapour Pressures of Various Aqueous Solutions*

The preceding results, obtained with various organic compounds, made it obvious that substances of this class may deviate so markedly from the laws of solutions and be so easily affected when added to other solutions, that they are unsuited for determining the state of water in solution. Sucrose might be expected to behave equally anomalously and it is unfortunate that Newton and Gortner (23) adopted this substance for studying water binding. Because of the prominence which the results obtained by their method has received, a further study has been made of the vapour pressures of solutions, with and without the addition of sucrose, to determine to what extent the results are valid for interpreting the condition of the water in such solutions.

Sucrose is normally believed to be hydrated in solution (25) so that it is necessary to correct any observed readings of solutions to which sucrose is added for the water of hydration removed by the sucrose from the solution. Although Newton and Gortner were aware of this hydration and corrected for its effect on the freezing point depression of the sucrose, they failed to realise the equal effect of this hydration in increasing the freezing point depression of the substances in the solution being studied. This source of error in Newton and Gortner's method has been recently pointed out by Moran and

Smith (21). The formula used by Newton and Gortner (6) for determining bound water is:

$$\text{Percentage of bound water} = \frac{\Delta_a - (\Delta + K_m)}{\Delta_a - \Delta} \times 89.2$$

in which  $\Delta_a$  is the freezing point depression observed on adding 0.01 mole of sucrose to a quantity of the solution, which is being investigated, containing 10 gm. of water:  $\Delta$  is the freezing point depression of the solution investigated:  $K_m$  that of the sucrose solution added (corrected for its hydration) which in the case of the molar solutions used, equals  $2.085^\circ$ , and 89.2 is the percentage of "free" water in a molar solution of sucrose. The added sucrose should, if we assume it to be hydrated, remove six molecules (108 gm.) of water from each 1000 gm. of total water present in the solution, thereby increasing the freezing point depression of the solution,  $\Delta$ , to  $\frac{1000}{892} \Delta$ . The latter term should, therefore, be introduced, in place of  $\Delta$  into the above formula of Newton and Gortner, which now becomes

$$\text{Percentage of "bound" water} = \frac{\Delta_a - \left( \frac{1000}{892} \Delta + K_m \right)}{\Delta_a - \frac{1000}{892} \Delta} \times 89.2$$

This modification of Newton and Gortner's method of calculation will markedly affect results quoted by these authors in which  $\Delta$  is appreciable compared to  $\Delta_a$  and  $K_m$  as is the case in practically all of the substances studied by these authors with the exception of gum acacia. In the latter case, the correction is comparatively slight.

Newton and Gortner's results by the above method, as applied to the juices of the wheat plant (*Triticum vulgare*) led these authors to conclude that winter hardness in such plants is related to the amount of bound water present in different varieties. If one corrects their results by the use of the modified formula described above, one finds very little evidence to substantiate this theory. Thus in the case of *Triticum vulgare* var. *super*, in which 4.4 per cent of the water was found to be bound, recalculation shows the results to indicate that all the water is actually free. Recalculation of the results for a second

species of the same plant shows—3.6 per cent of the total water to be bound (an obviously impossible result) instead of 0.9 per cent as given by Newton and Gortner. The above two varieties of wheat were non-hardy, but in the case of *Triticum vulgare* var. *Minhardi*, a bardy species, recalculation also shows a “negative” amount of “bound” water to be present instead of 2.2 per cent as claimed.

Obviously, then, the method employed by Newton and Gortner is unsuited for the determination of the amount of bound water in solutions and leads, in many cases, to the impossible conclusion that a negative amount of water is actually bound. It might be objected that

TABLE VI

*The Vapour Pressure Lowerings Observed on Adding Various Substances to a Molar Sucrose Solution as Compared to Those Observed When These Substances Are Present in Equal Concentration in a Pure Aqueous Solution*

| Substance added    | Molality in final solution | Molality of sucrose in final solution | Ratio of vapour pressure lowering produced by substance added to that produced by adding same substance to H <sub>2</sub> O |
|--------------------|----------------------------|---------------------------------------|---|
| NaCl.....          | 0.07                       | 1.0                                   | 1.12  |
| NaCl.....          | 0.75                       | “                                     | 1.00  |
| KCl.....           | 0.05                       | “                                     | 1.05  |
| KCl.....           | 0.093                      | “                                     | 1.00  |
| Urea.....          | 0.15                       | “                                     | 0.90  |
| Urea.....          | 0.3                        | “                                     | 0.80  |
| Lactic acid.....   | 0.04                       | “                                     | 1.18  |
| Succinic acid..... | 0.1                        | “                                     | 1.20  |

the results of Newton and Gortner are rendered invalid by the method employed by these authors (freezing point measurements) which may be subject to great error in the fluids studied. Application of vapour pressure determinations lead one, however, to the same conclusion, viz., that sucrose, like the other organic substances described in the preceding section, is unfit for determining the state of water in solutions, except possibly in certain unpredictable cases.

A series of results obtained during the present study is given in Table VI. They demonstrate the complexity of the conditions existing in solutions containing sucrose and the marked deviations from the



simple behaviour assumed by Newton and Gortner. Nor is there any apparent generalization by which one may predict the lowering obtained on adding sucrose (sufficient to make a molar solution) to simple solutions. In the case of more dilute solutions (0.05 molar sucrose), the conditions, as will be subsequently shown, are more easily interpreted.

The result (Table VI) obtained on adding NaCl (to make a 0.07 molar solution) to molar sucrose is compatible with the usually accepted view that sucrose is hydrated with the formation of a hexahydrate. When such a solution is 0.75 molar with respect to NaCl, on the other hand, the results would indicate either the complete absence of any water of hydration or the dissociation of part of such water from the sucrose hydrate. This dissociation would be only partial, for were it complete, the observed lowering of the *NaCl-sucrose* solution would have been less than the sum of the lowerings due to NaCl and sucrose when dissolved separately, since the liberated water of hydration by diluting the sucrose as well as acting as solvent for the NaCl would diminish the vapour pressure lowering exerted by the former.

In the case of KCl added to molar sucrose, one obtains results analogous to those with NaCl, but differing quantitatively from the latter. A 0.05 molar KCl solution indicates the presence of 5 per cent "bound" water which corresponds approximately to the formation of a sucrose trihydrate instead of the hexahydrate indicated by the stronger (0.07 molar) NaCl solution. The two salts obviously differ in their action in sucrose solutions. A 0.093 molar solution of KCl gave no indication (as neither did the 0.75 molar NaCl solution) of hydrate formation. The latter result is in accord with the electromotive force measurements of Corran and Lewis (2) who demonstrated that KCl is apparently soluble in the water of hydration of sucrose, which is tantamount to saying that the *KCl-sucrose* solution behaves as if no water of hydration existed. These authors did not investigate solutions less than 0.1 molar in respect to KCl in which, according to the results of Table VI, water of hydration is still apparent.

One can attribute the above results with *NaCl* and *KCl-sucrose* solutions to the formation of complexes of the type  $C_{12}H_{22}O_{11} \cdot NaCl \cdot 2H_2O$ , such as have been demonstrated to occur by Schoolt (27).

The anomalous results shown by the organic substances, urea, lactic acid, and succinic acid, have been discussed in the preceding section. It is most probable that they undergo marked changes—dissociation or association—in the presence of sucrose and consequently give the results shown in Table VI. Any attempt to calculate the degree of hydration of sucrose by the use of these substances would lead to the view that either a decahydrate of sucrose exists (data of lactic and succinic acids) or that a “negative” quantity of water is bound. Conversely, using sucrose to determine the hydration of urea, lactic acid or succinic acid solutions would lead to similar conclusions regarding these substances. In the case of biological fluids we deal for the most part with solutions of organic substances of the nature of urea, lactic acid, succinic acid, etc., and in such cases the use of molar sucrose, as in the method of Newton and Gortner, leads to erroneous results. The “negative” values for the amount of water bound in the expressed juice of wheat, as calculated above from the data of Newton and Gortner, are quite explicable when we consider the results of Table VI which deal with organic substances dissolved in molar sucrose.

## VI

### *The State of Water in Several Colloidal and Inorganic Solutions*

In the view of the above findings, it appeared that the substances most likely to be suitable for determining hydration would be inorganic salts such as NaCl or KCl. These two substances are particularly appropriate for the investigation of biological material. Their natural presence in such material would make it probable that a further addition of a small quantity of them would not produce any marked effect on such biological systems. NaCl and KCl are also desirable because of the absence of any gross hydration of their ions. For the purpose of the present study, the following substances were selected—gelatin and gum acacia, as examples of hydrophilic colloids, which according to the claims of many “bind” water to a great extent; and LiCl and CaCl<sub>2</sub> whose cations are strongly hydrated judging by their hygroscopicity and the deviation of their colligative properties from the theoretically calculated values.

The terms “hydration” and “water binding” have been used inter-

changeably by many authors, the latter term being used perhaps in a less well defined sense than the former. There seems to be no reason, however, for such a division in regard to the manner in which water may be combined with a solute. Whether such water be actually united chemically with the formation of compounds amenable to stoichiometric representation, or whether the water be merely adsorbed in the sense in which a dye is adsorbed on proteins in solution, such water will differ from the remainder of the solvent in its physical

TABLE VII

*The Apparent Hydration of Several Hydrophilic Colloids as Determined by the Colligative Method*

| Substance added to solution       | Gelatin (1 per cent solution at pH = 7.0)    |  | Apparent hydration of the colloid in the original solution |
|-----------------------------------|--|--|--|
|                                   | Molality of substance added in final mixture | Ratio of vapour pressure lowering produced by the added substance to its lowering when dissolved in pure water |  |
|                                   | moles per 1000 gm. of H <sub>2</sub> O       |  | gm. of H <sub>2</sub> O per gm. of colloid                 |
| NaCl.....                         | 0.05   | 1.01   | 1.0  |
| KCl.....                          | 0.05   | 1.03   | 3.0  |
| Sucrose.....                      | 0.1  | 1.03   | 3.0  |
| Gum acacia (10 per cent solution) |  |  |  |
| NaCl.....                         | 0.05   | 1.00   | 0  |
| KCl.....                          | 0.07   | 1.00   | 0  |
| Sucrose.....                      | 0.043  | 1.005  | 0.05   |
| “ .....                           | 0.09   | 1.05   | 0.5  |
| “ .....                           | 0.18   | 1.07   | 0.7  |

and chemical properties. We shall therefore use the term “hydration” in the subsequent part of this paper to include the usual implications of the term “water binding” and *vice versa*.

### *Gelatin*

The results obtained by the present method for gelatin are given in Table VII. The results with KCl and sucrose agree in indicating an hydration of gelatin of about 3 gm. of water per gram of gelatin. The results for NaCl, however, indicate a lower hydration. Such

hydration of gelatin has been demonstrated by a number of previous observers (15, 28) but the magnitude of the hydration as found in the present study is comparatively small. The results are in closer agreement with those of Moran (19) and Adair and Callow (1) than they are with those of Kunitz (15). The method of Kunitz, however, differs from that used in the present work in that any water transported with the gelatin particles either by capillarity or other mechanical means would be determined as water of hydration. Such mechanically carried water would exert a normal vapour pressure and hence appear as normal solvent when investigated by the method used in the present study. The addition of salts affects the hydration of gelatin and the discrepancy between the results obtained with KCl and NaCl may be attributed to their difference in action in this respect or to a greater combination of the NaCl with gelatin (31) as compared to KCl.

The question of the effect of passage of a gelatin solution from the *sol* to the *gel* form on the state of water in such solutions is of interest. The hydration of a *gel* formed from a 5 per cent gelatin solution was found to be approximately that of the *sol* form calculated for the same concentration. This is in agreement with the conclusion of Svedberg (28). By saturating filter paper with the *sol* and placing it on the thermopiles where gelation occurred, it is possible to determine the vapour pressure of *gels* by Hill's method in a manner similar to that by which solutions are studied. A preliminary experiment on human blood also indicated that the process of clotting was not accompanied by any detectable change in vapour pressure. These results indicate that we cannot picture the process of gelation as involving any combination of colloid with water but that the latter is merely held, in its usual state, in the interstices of the *gel* fibrillae.

#### *Gum Acacia*

Gum acacia may be taken as a typical hydrophilic colloid. Its apparent hydration is given in Table VII. It will be observed that the experiments both with NaCl and with KCl fail to indicate any hydration of this substance. The results with sucrose (which as was shown above are of little value for determining hydration) vary considerably with change in the concentration of the sucrose. In a

0.18 molar sucrose solution, gum acacia apparently behaves as if each gram of the substance combined with 0.7 gm. of water, a value equal to that found by Newton and Gortner (6) for acacia at  $0^{\circ}$  when sucrose sufficient to make a molar solution was added. As we decrease the concentration of sucrose, the apparent degree of hydration rapidly diminishes; and only 0.05 gm. of water appears to be combined with each gram of acacia when we utilise a 0.043 molar sucrose solution for our determination. Obviously then the amount of sucrose added determines the result obtained, which is what might be expected from the anomalous behaviour of sucrose as described above. It is only with dilute solutions (0.05 molar) of sucrose that accurate results approximating those obtained by the use of other substances are obtained. Theoretically it is to be expected that the most accurate data (as regards furnishing information concerning the state of water) will be obtained when the least possible quantity of a substance is added to the solution in question. For in the case of infinitely dilute solutions the activities of substances in mixed solutions are most likely to be the same as their activities when present alone in solution (16). Unfortunately our knowledge concerning the behaviour of moderately concentrated mixtures is still too inadequate to permit the use of such mixtures for our present problem. It is obvious, however, that we must consider not only the possible interaction of electrolytes in mixed solutions but the interaction of electrolytes with non-electrolytes, as well, as has been pointed out by Scatchard (26).

The results obtained in the present work with gum acacia and gelatin, those of Hill (11) on blood, those of Svedberg (29) using his method of ultracentrifugalisation, those of Moran (19, 20) obtained by a dilatometric method, all give evidence for the view that hydrophilic colloids are not hydrated to a great extent as has been claimed by many authors. It is well known that certain solid colloidal substances (e.g. silica gel) are capable of adsorbing appreciable quantities of water and it is probable that certain hydrophilic colloids in solution combine with water as a result of the residual affinities which these substances possess. In the case of gelatin the amount so held is appreciable, but in the case of the blood proteins, gum acacia, etc., the amount is undetectable.

*Lithium and Calcium Chlorides*

It is generally agreed that the lithium ion is strongly hydrated and this hydration was therefore investigated by the present method. The results, as given in Table VIII, show variations which far exceed the experimental errors of the methods employed. Thus, whereas a mixture 0.05 molar with respect to NaCl would indicate that 13 per cent of the water in a 1.33 molal LiCl solution is bound to the solute, the result with KCl shows only 4 per cent to be bound. The results with sucrose, if corrected as shown in Section IV of this paper,

TABLE VIII

*The Apparent Hydration of LiCl and CaCl<sub>2</sub> as Determined by the Colligative Method.*

| Substance added to solution     | Molality of substance added in final mixture | Ratio of vapour pressure lowering produced by the added substance to its lowering when dissolved in pure water | Apparent hydration of the solute in the original solution |
|---------------------------------|--|--|---|
| LiCl (1.33 molar)               |  |  |   |
|                                 | moles per 1000 gm. of H <sub>2</sub> O       |  | moles of H <sub>2</sub> O per mole of solute              |
| NaCl.....                       | 0.05   | 1.13   | 5.4   |
| KCl.....                        | 0.05   | 1.04   | 1.7   |
| Sucrose.....                    | 0.05   | 1.06   | —   |
| “ .....                         | 0.1  | 1.11   | —   |
| CaCl <sub>2</sub> (0.236 molar) |  |  |   |
| NaCl.....                       | 0.05   | 1.075  | 17  |
| KCl.....                        | 0.05   | 1.00   | 0   |
| Sucrose.....                    | 0.1  | 1.065  | —   |

lead to values which depend upon the concentration of sucrose employed.

Results, equally dependent on the nature of the substance added to the solution investigated and its concentration, were obtained with CaCl<sub>2</sub> as shown in Table VIII. The results obtained with the three substances NaCl, KCl, and sucrose, differ markedly. The apparent absence of any hydration of CaCl<sub>2</sub> when KCl is added to CaCl<sub>2</sub> may be explained as due to a partial dissociation of the CaCl<sub>2</sub>-H<sub>2</sub>O complex, such as was described in Section IV in discussing sucrose-

0.18 molar sucrose solution, gum acacia apparently behaves as if each gram of the substance combined with 0.7 gm. of water, a value equal to that found by Newton and Gortner (6) for acacia at  $0^{\circ}$  when sucrose sufficient to make a molar solution was added. As we decrease the concentration of sucrose, the apparent degree of hydration rapidly diminishes; and only 0.05 gm. of water appears to be combined with each gram of acacia when we utilise a 0.043 molar sucrose solution for our determination. Obviously then the amount of sucrose added determines the result obtained, which is what might be expected from the anomalous behaviour of sucrose as described above. It is only with dilute solutions (0.05 molar) of sucrose that accurate results approximating those obtained by the use of other substances are obtained. Theoretically it is to be expected that the most accurate data (as regards furnishing information concerning the state of water) will be obtained when the least possible quantity of a substance is added to the solution in question. For in the case of infinitely dilute solutions the activities of substances in mixed solutions are most likely to be the same as their activities when present alone in solution (16). Unfortunately our knowledge concerning the behaviour of moderately concentrated mixtures is still too inadequate to permit the use of such mixtures for our present problem. It is obvious, however, that we must consider not only the possible interaction of electrolytes in mixed solutions but the interaction of electrolytes with non-electrolytes, as well, as has been pointed out by Scatchard (26).

The results obtained in the present work with gum acacia and gelatin, those of Hill (11) on blood, those of Svedberg (29) using his method of ultracentrifugalisation, those of Moran (19, 20) obtained by a dilatometric method, all give evidence for the view that hydrophilic colloids are not hydrated to a great extent as has been claimed by many authors. It is well known that certain solid colloidal substances (*e.g.* silica gel) are capable of adsorbing appreciable quantities of water and it is probable that certain hydrophilic colloids in solution combine with water as a result of the residual affinities which these substances possess. In the case of gelatin the amount so held is appreciable, but in the case of the blood proteins, gum acacia, etc., the amount is undetectable.

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# THE ISOELECTRIC POINT OF A STANDARD GELATIN PREPARATION<sup>1</sup>

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## I

### INTRODUCTION

The isoelectric point of an amphoteric substance may be defined as the hydrogen ion activity of a solution or suspension in which the ampholyte shows no migration in an electric field. This definition, which is quite generally accepted, is in accord with the original use of the term by Hardy (1899-1900), as well as with the definitions given by Michaelis (1922) and Sørensen, Linderstrøm-Lang and Lund (1925-28). Michaelis defines the isoelectric point, for the most general case which he considers, as the value of  $[H^+]$  at which equivalent amounts of positive and negative ampholyte ions are present. Sørensen and his coworkers define it as the hydrogen ion activity at which the mean valency of the ampholyte is zero. Obviously these theoretical definitions must correspond to the above experimental definition in terms of zero velocity of cataphoresis.

The possibility that the value of the isoelectric point may vary with the presence of ions other than  $H^+$  and  $OH^-$  has been considered by Sørensen and his coworkers (1925-28). If some other negative ion, for example, were combined with or adsorbed by the ampholyte, giving it a negative charge, it might well be true that this charge could be neutralized, and the ampholyte rendered isoelectric, only at a more

<sup>1</sup> Presented before the American Society of Biological Chemists, Montreal, April 10, 1931.

<sup>2</sup> Most of the experimental work was done by Miss Ruth C. Belden. A few of the earlier measurements were carried out by Miss Esther R. Mason and by Dr. Rubert S. Anderson.

acid pH than that corresponding to the isoelectric point in the absence of such foreign ions. In order for the isoelectric reaction to be completely defined, it may be necessary to specify the concentrations of all substances in the system, or the activities of all ions, as well as that of  $H^+$ . Owing to the more marked effect of  $H^+$  and  $OH^-$  on the charge of ampholytes, it seems reasonable to keep the definition of the isoelectric point in terms of  $[H^+]$  or pH, recognizing that it may not be a constant quantity for any particular ampholyte, but that it may vary with the presence of other ions.

In the case of gelatin the isoelectric point was first determined by Michaelis and Grineff (1912), who located it as between  $[H^+] = 1.6 \times 10^{-5}$  and  $3.5 \times 10^{-5}$ , average,  $2.5 \times 10^{-5}$ . In terms of pH these figures correspond to 4.80, 4.46, and 4.60. Most subsequent determinations of this quantity for gelatin have been more or less indirect, and in the few cases where cataphoresis measurements have been made the isoelectric point has not been very exactly located. Thus Loeb (1922) interpreted his varied observations on physical and chemical properties of gelatin as indicating an isoelectric point at pH 4.7, but Kraemer and Dexter (1927) showed that very few if any of the earlier observations were inconsistent with an isoelectric point at pH 5.0, which they located quite exactly as the pH of the maximum light scattering (Tyndall effect) for calfskin gelatins. They showed, however, that the figures obtained depended considerably on the source and method of preparation of the gelatin. The writer (1928-29) found that a gelatin sample from the same source as that used by Loeb, purified according to Northrop and Kunitz (1927-28), had minimum osmotic pressure and maximum opacity at pH  $5.05 \pm 0.05$ , which was also the pH of solutions of this gelatin in water.

Some of the discrepancies in previous results are probably due to differences in the gelatins used, and others to the more or less indirect nature of the methods. The present paper reports a study of the isoelectric point of samples prepared according to definite specifications of a committee (Davis, Sheppard, and Briefer, 1929; Hudson and Sheppard, 1929) of the Leather and Gelatin Division of the American Chemical Society; hence it should be possible for workers in other laboratories to obtain identical material. The isoelectric point of such gelatin has already been reported by Sheppard and

Houck (1930) to be at  $\text{pH } 4.9 \pm 0.10$  by electric migration, and at  $\text{pH } 4.9 \pm 0.05$  both by light scattering and by alcohol precipitation. The results of the present work confirm those of these workers within their limits, but since somewhat different methods were used, and since the results permit of a more precise location of the isoelectric point, it is believed that a detailed report of the work may be of interest.

The gelatin used was prepared, according to the specifications mentioned, in the laboratories of the Eastman Kodak Company.<sup>3</sup>

## II

### *Isoelectric Point from pH Values of Pure Gelatin Solutions*

It was shown by Sørensen (1912) that the pH of a pure solution of a simple ampholyte in water must lie between that of pure water and that of the isoelectric point of the ampholyte, approaching the latter figure as the concentration is increased. Thus he calculated that for a molar solution of glycine the pH should differ from that of the isoelectric point by only 0.001 pH. Similar considerations ought to hold for a colloidal ampholyte like gelatin or any other protein, even though it may not be possible to calculate the pH, as Sørensen did, from the values of the ionization constants. Hence it seemed reasonable to measure the pH of a series of gelatin solutions of different concentrations, with the expectation that the values would approach a constant figure, that of the isoelectric point, as the concentration was increased.

The pH measurements were made with hydrogen electrodes at 30°C., using a KCl-agar junction. The pH values are based on the figure 1.075 for 0.1000 molal HCl, liquid junction potentials being assumed constant.

The first measurement of a 1 per cent solution (Eastman Standard Gelatine, Lot 1) yielded the surprisingly low value of pH 4.61. This was found to be due to an acid, presumably acetic, which was quite completely removed from the gelatin by further washing of a 5 gm. sample with 4 changes, about 1 liter each, of cold distilled water. After such washing, the gelatin gave solutions of higher pH values, which were, for concentrations of 1, 2, 5, and 12 gm. per 100 cc., 4.90,

<sup>3</sup> The writer is indebted to Dr. S. E. Sheppard for two samples of "Eastman Standard Gelatine."

4.88, 4.87, and 4.86 respectively. These values are shown graphically in Fig. 1, in which the pH values are plotted against the reciprocals of the gelatin concentrations in gm. per 100 cc. The value obtained from this figure by extrapolation to infinite concentration of gelatin is pH  $4.86 \pm 0.01$ , and it is this value which is inferred to be the isoelectric point of the gelatin.

This method for determining the isoelectric point, as well as that used in the following section, implies that the ampholyte used must be pure, or at least free from appreciable amounts of ions capable of

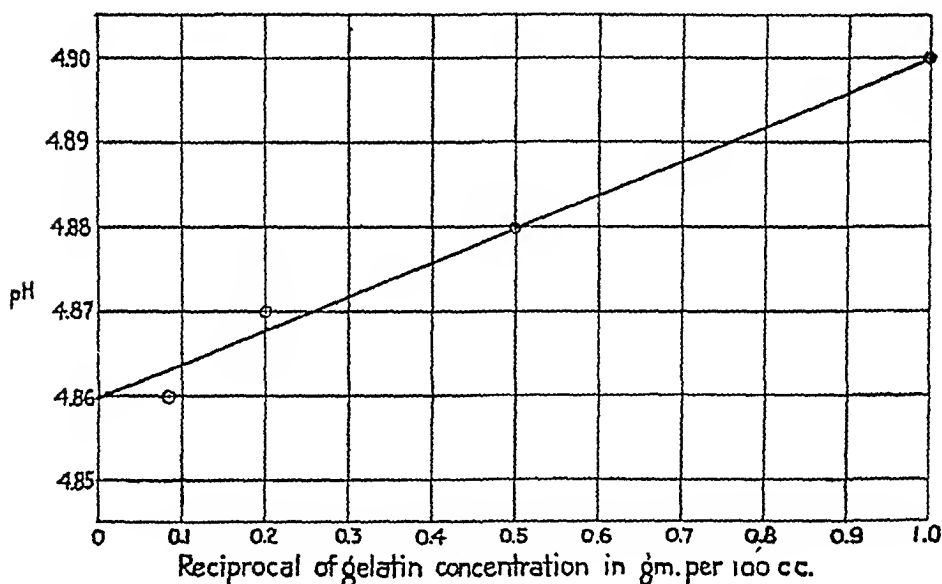


FIG. 1. pH values of solutions of standard gelatin in distilled water as a function of the reciprocal of the gelatin concentration. By extrapolation to infinite concentration, the pH of the isoelectric point is estimated as  $4.86 \pm 0.01$ .

combining with it or altering its charge. Loeb (1922) has pointed out that a protein is most readily obtained in such a state of purity by washing or dialysis at the pH of its isoelectric point. The method used in purifying the gelatin used in these experiments involves washing at about pH 4.7, which is not the value obtained for the isoelectric point either of this gelatin or of that studied in the writer's former work (1928-29). Theoretically it should be possible to remove the last traces of impurities by prolonged washing with water, since proteins

are weak electrolytes and their salts should therefore be hydrolyzable. That the washing of the gelatin here studied actually had this effect is indicated by the low ash content of the material, which was 0.04 to 0.05 per cent, as well as its low specific conductivity, which was  $3.8 \times 10^{-5}$  reciprocal ohms for a 5.6 per cent solution at 30°C. and  $5.0 \times 10^{-5}$  for a 9.4 per cent solution. Moreover the methods of Sections II and III of this paper, which depend on the initial purity of the gelatin, gave results in agreement with those of Sections IV and V, which do not require that the gelatin be absolutely pure at the start.

### III

#### *Isoionic Point by pH Values of Weakly Buffered Gelatin Solutions*

Sørensen (1912) pointed out that the amount of acid or base required to bring a solution of an ampholyte to its isoelectric point should be independent of the ampholyte concentration and identical with the amount required to bring an equal volume of pure water to the same pH. He later (1917) applied this method to the determination of the isoelectric point of carefully purified egg albumin. In a still later paper Sørensen, Linderstrøm-Lang, and Lund (1925-28) showed that this method would not necessarily give the isoelectric point if the ampholyte were capable of combining with ions other than hydrogen or hydroxyl. Accordingly they defined a new quantity, the isoionic reaction, as the hydrogen ion activity<sup>4</sup> at which the quantity of acid or base combined with the ampholyte is zero. It is the isoionic rather than the isoelectric reaction which is given by this method of Sørensen.

The method was slightly modified by Michaelis (1912), who determined the isoelectric points of the soluble ampholytes phenyl alanine and glycocoll by measuring the pH of dilute acetate buffers with and without the ampholyte. On the acid side of its isoelectric point the pH of the buffer was raised by the ampholyte, on the alkaline side it was lowered. Michaelis was thus able to locate the isoelectric point within a few tenths of a pH unit.

It was suggested by Sheppard (1929) that this buffer method might

<sup>4</sup> Sørensen's distinction between pH and p<sub>a</sub>H is here disregarded, as the standard of pH used in this paper is based on an activity coefficient for HCl and not on conductivity measurements, as was Sørensen's original definition of pH.

well be applied to gelatin. Accordingly measurements were made of pH in acetate buffers of varied concentration, made up in each case with and without gelatin. The gelatin used in these experiments (Eastman Standard Gelatine, Lot 48) had been more thoroughly washed at the time of preparation, and no appreciable amounts of acid could be removed from it by further washing. The pH measurements in this case were made at 30°C. with the hydrogen electrode apparatus of Simms (1923), in which contact is made with saturated KCl in an open stop-cock. The standard used was again pH 1.075 for 0.1000 molal HCl. The results are given in Table I.

To find the isoionic point of the gelatin, curves were obtained by plotting the changes in pH produced by the gelatin against the pH of buffer solutions of the same concentration without gelatin. Fig. 2, which shows the data of Experiments 3 and 7 of Table I, is illustrative of the nature of all the curves, the others being equally smooth. For each experiment the isoionic point is given by the intersection of the curve with the line of zero change in pH. The figures so obtained are given in the last column of Table I. Their mean value is 4.85, with an average deviation of 0.01. It is to be noted that this value is independent of the gelatin concentration within the limits studied (1 to 4 per cent) and of the salt concentration up to an ionic strength of 0.105 (Experiment 7). Hence it may be concluded that within these limits the isoionic point of this gelatin corresponds to  $\text{pH } 4.85 \pm 0.01$ .

#### IV

##### *Isoelectric Point by Maximum Turbidity of Gelatin Gels*

While the definition of the isoelectric point says nothing about turbidity, it is generally accepted that an acidity corresponding to the isoelectric point produces a maximum of light scattering in gelatin gels. In a previous study (1928-29) of another gelatin preparation, it was found that the pH of maximum opacity was identical with that of minimum osmotic pressure, and the latter value has been shown by Loeb (1922) to be theoretically identical with the isoelectric point. Accordingly it seemed worth while to investigate the behavior of the standard gelatin with respect to turbidity. The method adopted was

TABLE I

*Isoionic Reaction of Standard Gelatin by pH of Acetate Buffers with and without Gelatin*

| Exp. no. | Normality of Na acetate in buffer | Gelatin per 100 cc. | pH of buffer without gelatin | pH of buffer with gelatin | Change in pH due to gelatin | pH of isoionic reaction |
|----------|-----------------------------------|---------------------|------------------------------|---------------------------|-----------------------------|-------------------------|
|          |                                   | gm.                 |                              |                           |                             |                         |
| 1        | 0.001                             | 1.0                 | 4.47                         | 4.70                      | +0.23                       | 4.84                    |
|          |                                   |                     | 4.67                         | 4.79                      | +0.12                       |                         |
|          |                                   |                     | 4.85                         | 4.86                      | +0.01                       |                         |
|          |                                   |                     | 5.08                         | 4.90                      | -0.18                       |                         |
|          |                                   |                     | 5.24                         | 4.95                      | -0.29                       |                         |
| 2        | 0.001                             | 2.0                 | 4.47                         | 4.75                      | +0.28                       | 4.85                    |
|          |                                   |                     | 4.67                         | 4.81                      | +0.14                       |                         |
|          |                                   |                     | 4.85                         | 4.85                      | 0.00                        |                         |
|          |                                   |                     | 5.08                         | 4.88                      | -0.20                       |                         |
|          |                                   |                     | 5.24                         | 4.90                      | -0.34                       |                         |
| 3        | 0.001                             | 4.0                 | 4.47                         | 4.80                      | +0.33                       | 4.86                    |
|          |                                   |                     | 4.67                         | 4.83                      | +0.16                       |                         |
|          |                                   |                     | 4.85                         | 4.86                      | +0.01                       |                         |
|          |                                   |                     | 5.08                         | 4.87                      | -0.21                       |                         |
|          |                                   |                     | 5.27                         | 4.87                      | -0.40                       |                         |
| 4        | 0.005                             | 1.0                 | 4.03                         | 4.19                      | +0.16                       | 4.86                    |
|          |                                   |                     | 4.26                         | 4.39                      | +0.13                       |                         |
|          |                                   |                     | 4.47                         | 4.56                      | +0.09                       |                         |
|          |                                   |                     | 4.65                         | 4.71                      | +0.06                       |                         |
|          |                                   |                     | 4.86                         | 4.86                      | 0.00                        |                         |
|          |                                   |                     | 5.05                         | 4.98                      | -0.07                       |                         |
|          |                                   |                     | 5.27                         | 5.09                      | -0.18                       |                         |
|          |                                   |                     | 5.52                         | 5.18                      | -0.34                       |                         |
|          |                                   |                     | 5.74                         | 5.24                      | -0.50                       |                         |
| 5        | 0.005                             | 2.0                 | 4.47                         | 4.66                      | +0.19                       | 4.88                    |
|          |                                   |                     | 4.65                         | 4.77                      | +0.12                       |                         |
|          |                                   |                     | 4.86                         | 4.86                      | 0.00                        |                         |
|          |                                   |                     | 5.05                         | 4.94                      | -0.11                       |                         |
|          |                                   |                     | 5.27                         | 4.99                      | -0.28                       |                         |
| 6        | 0.005                             | 4.0                 | 4.44                         | 4.66                      | +0.22                       | 4.84                    |
|          |                                   |                     | 4.63                         | 4.75                      | +0.12                       |                         |
|          |                                   |                     | 4.83                         | 4.83                      | 0.00                        |                         |
|          |                                   |                     | 4.99                         | 4.89                      | -0.10                       |                         |
|          |                                   |                     | 5.25                         | 4.94                      | -0.31                       |                         |



TABLE I—*Concluded*

| Exp. no.     | Normality of Na acetate in buffer | Gelatin per 100 cc. | pH of buffer without gelatin | pH of buffer with gelatin | Change in pH due to gelatin | pH of isoionic reaction |
|--------------|-----------------------------------|---------------------|------------------------------|---------------------------|-----------------------------|-------------------------|
| 7            | 0.005, in 0.1 <i>N</i> KCl        | <i>gm.</i><br>1.0   | 4.37                         | 4.53                      | +0.16                       | 4.84                    |
|              |                                   | 4.56                | 4.67                         | +0.11                     |                             |                         |
|              |                                   | 4.77                | 4.80                         | +0.03                     |                             |                         |
|              |                                   | 4.97                | 4.91                         | −0.06                     |                             |                         |
|              |                                   | 5.21                | 5.01                         | −0.20                     |                             |                         |
| Average..... |                                   |                     |                              |                           |                             | 4.85                    |

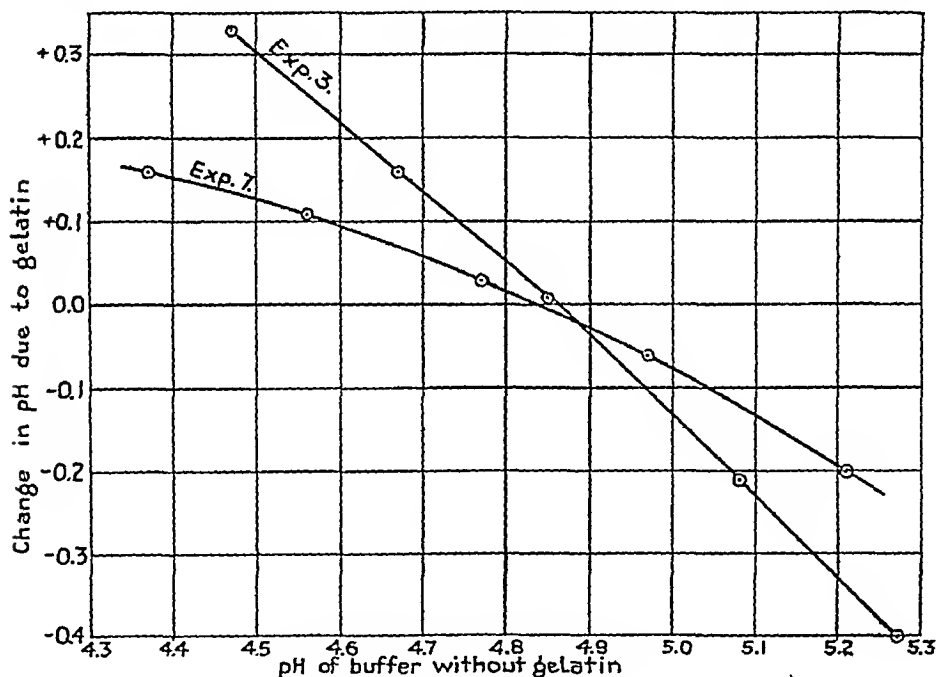


FIG. 2. Change in pH of acetate buffers, produced by gelatin in solution, as a function of pH of buffers of the same concentration without gelatin. These curves show the data for Experiments 3 and 7 (Table I). The isoionic point is given by the intersections with the line of zero change, and is at  $\text{pH } 4.85 \pm 0.01$ .

the simple one described in the previous paper, which consists simply in the inspection in ordinary daylight of samples of gelatin solutions of equal volume and varied pH which have been allowed to set in uniform test tubes in a refrigerator. The pH values were determined at  $30^\circ\text{C}$ . after melting the gels in warm water.

In the first experiment Lot 1 of the standard gelatin was used, after the excess acid had been removed by washing, as already described. The concentration of the gelatin was 1 gm. per 100 cc., and the pH was varied by the addition of very dilute HCl. The gel showing maximum opacity was in 0.0001 N HCl and had a pH value of 4.85, while those adjacent in the series were in 0.00005 and 0.00015 N HCl, with pH values of 4.86 and 4.82 respectively. Before this gelatin was washed free from acid, the maximum turbidity of 0.2 per cent and 1.0 per cent solutions was found to be at pH 4.85 and 4.87 ( $\pm 0.05$ ) respectively, the tubes in this case requiring the addition of dilute NaOH to produce these pH values.

In the second experiment the gelatin used was from Lot 48. The pH was varied by acetic acid in 0.001 N sodium acetate, the solutions being those of Experiments 2 and 3 in Table I. In each case the turbidity was at a maximum in the middle solution of the series, so that from these observations the pH of maximum turbidity may be placed at  $4.85 \pm 0.03$ .

## v

*Isoelectric Point by Electrical Migration*

By definition the most direct way to determine the isoelectric point is by determining the pH corresponding to zero migration in an electric field. Previous determinations in the case of gelatin have been made by the macroscopic U-tube method of cataphoresis, and the results, as already mentioned, have not defined the isoelectric point very exactly. Since it had been shown by Loeb (1922-23b) that collodion particles suspended in a gelatin solution behaved in migration experiments as if they were particles of gelatin, it seemed likely that such suspensions might be used for an exact determination of the isoelectric point. Similar determinations have been made in the case of egg albumin by Abramson (1928), who used quartz particles which behaved as if coated with the protein.

The migration was conducted in a cell of the type described by Northrop and Kunitz (1924-25, see also Mudd, 1928), the source of potential being a radio "B" battery of nominally 135 volts. Observations were made with a microscope equipped with an 8 mm. objective

TABLE I—*Concluded*

| Exp. no.     | Normality of Na acetate in buffer | Gelatin per 100 cc. | pH of buffer without gelatin | pH of buffer with gelatin | Change in pH due to gelatin | pH of isoionic reaction |
|--------------|-----------------------------------|---------------------|------------------------------|---------------------------|-----------------------------|-------------------------|
| 7            | 0.005, in 0.1 N KCl               | gm.<br>1.0          | 4.37                         | 4.53                      | +0.16                       | 4.84                    |
|              |                                   | 4.56                | 4.67                         | +0.11                     |                             |                         |
|              |                                   | 4.77                | 4.80                         | +0.03                     |                             |                         |
|              |                                   | 4.97                | 4.91                         | −0.06                     |                             |                         |
|              |                                   | 5.21                | 5.01                         | −0.20                     |                             |                         |
| Average..... |                                   |                     |                              |                           |                             | 4.85                    |

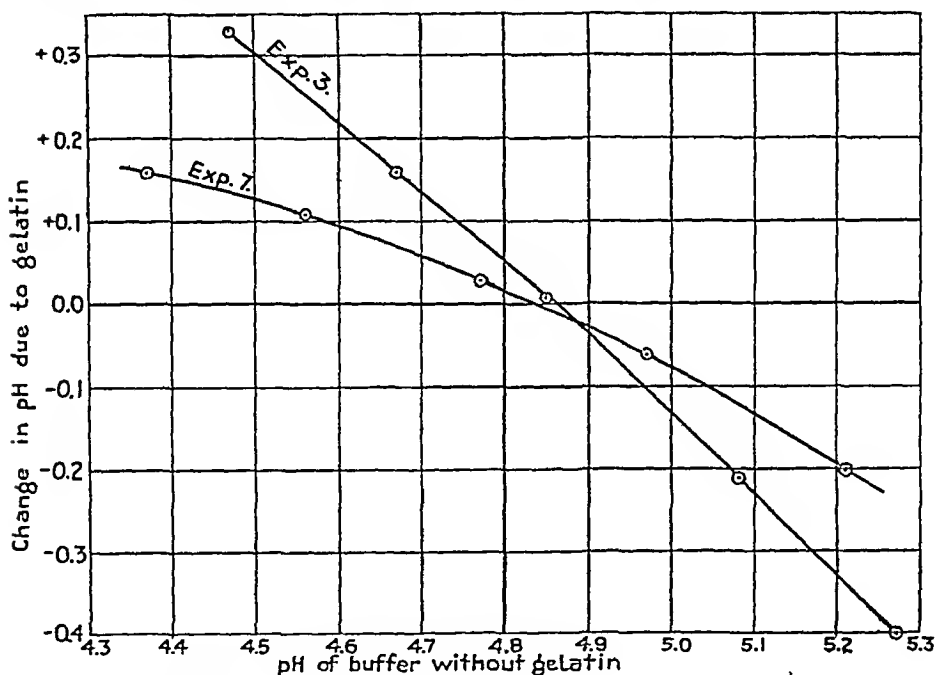


FIG. 2. Change in pH of acetate buffers, produced by gelatin in solution, as a function of pH of buffers of the same concentration without gelatin. These curves show the data for Experiments 3 and 7 (Table I). The isoionic point is given by the intersections with the line of zero change, and is at  $\text{pH } 4.85 \pm 0.01$ .

the simple one described in the previous paper, which consists simply in the inspection in ordinary daylight of samples of gelatin solutions of equal volume and varied pH which have been allowed to set in uniform test tubes in a refrigerator. The pH values were determined at  $30^\circ\text{C}$ . after melting the gels in warm water.

not been recalculated to unit potential gradient because the reproducibility of the observed velocities was much higher than that obtained in attempts to calibrate the cell by the methods described by previous workers (Northrop and Kunitz, 1924-25; Abramson, 1928-29). The potential drop in the cell, as obtained by the various methods of calibration, appeared to vary between 7 and 10 volts per cm. It is believed that these latter variations are not real, but are due to the inadequacy of the calibration. An approximate reduction of the observed velocities to unit potential gradient may be obtained by dividing them by 8.5.

Each experiment was run in duplicate with separate mixtures. The results are given in Table II.

The table shows that the agreement of pH values in the duplicate experiments was almost perfect, as might be expected for buffered solutions. The agreement of cataphoretic velocities, while less satisfactory, is such that the duplicate determinations may safely be averaged. The average velocities were plotted on a large scale against the average pH values for each concentration of sodium acetate, and a smooth curve was drawn by means of a flexible spline held by weights on all of the points. It was at first thought that the points of each experiment could best be represented by a straight line, but after all the data were plotted a consistent deviation from linearity was observed in every experiment. In order to get a curve to pass through all the points, it was necessary to draw it somewhat S-shaped. The curve of Fig. 3, which shows the results in a buffer 0.001 M with respect to sodium acetate, is typical. The points indicated by crosses were obtained from a separate experiment, not given in Table II, which was done after the curve was drawn. The data show no indication of a flattening of the curves at the isoelectric point, such as would be expected if the isoelectric region were not sharply defined.

From each experiment at a given salt concentration, the pH of the isoelectric point was obtained by the intersection of the curve with the line of zero velocity. The values found in this way are given in the last column of Table II, the average being pH 4.80. The data are probably not certain enough to warrant the inference of a trend in the values with salt concentration, although the values at the lower concentrations are slightly higher. It can only be concluded that the

cataphoretic isoelectric point of gelatin-coated collodion particles is at pH  $4.80 \pm 0.01$ , a value which differs by 0.05 from that obtained in

TABLE II

*Isoelectric Reaction of Standard Gelatin by Cataphoresis of Collodion Particles in 0.1 Per Cent Gelatin in Acetate Buffers*

Total E.M.F. = 133 to 137 volts.

Potential gradient in cell = 8.5 volts per cm. (approximate only).

Temperature, 20–22°C.

pH values at 30° by hydrogen electrode.

Algebraic sign of velocity is that of charge on particles.

| Conc. Na acetate | Ratio of acetic acid to Na acetate |                |      |                |      |                |      |                | Isoelectric point |
|------------------|------------------------------------|----------------|------|----------------|------|----------------|------|----------------|-------------------|
|                  | 1.85                               |                | 1.20 |                | 0.48 |                | 0.30 |                |                   |
|                  | pH                                 | $\mu$ per sec. | pH   | $\mu$ per sec. | pH   | $\mu$ per sec. | pH   | $\mu$ per sec. |                   |
| $\kappa$         |                                    |                |      |                |      |                |      |                | $pH$              |
| 0.001            | 4.52                               | +4.08          | 4.69 | +1.77          | 5.01 | -2.97          | 5.15 | -4.42          | 4.81              |
|                  | 4.53                               | +3.38          | 4.70 | +1.78          | 5.01 | -2.96          | 5.15 | -4.12          |                   |
|                  |                                    |                | 4.68 | +2.07          | 5.00 | -2.97          |      |                |                   |
| 0.003            | 4.47                               | +4.00          | 4.65 | +2.24          | 5.01 | -2.24          | 5.18 | -4.23          | 4.82              |
|                  | 4.46                               | +4.32          | 4.64 | +2.00          | 5.00 | -2.42          | 5.17 | -4.46          |                   |
| 0.005            | 4.44                               | +3.38          | 4.62 | +1.74          | 5.00 | -1.68          | 5.18 | -3.30          | 4.80              |
|                  | 4.44                               | +3.23          | 4.62 | +1.84          | 5.00 | -2.05          | 5.18 | -3.25          |                   |
| 0.010            | 4.41                               | +3.18          | 4.59 | +1.75          | 4.97 | -1.44          | 5.16 | -2.74          | 4.80              |
|                  | *4.40                              | +2.94          | 4.59 | +1.62          | 4.98 | -1.51          | 5.18 | -3.11          |                   |
| 0.020            | 4.39                               | +2.34          | 4.58 | +1.58          | 4.97 | -1.45          | 5.17 | -2.41          | 4.79              |
|                  | 4.40                               | +2.89          | 4.58 | +1.48          | 4.97 | -1.26          | 5.17 | -2.40          |                   |
| 0.030            | 4.45                               | +3.47          | 4.64 | +1.75          | 5.00 | -2.35          | 5.17 | -4.16          | 4.80              |
|                  | 4.47                               | +3.70          | 4.64 | +1.85          | 5.00 | -2.20          | 5.17 | -3.61          |                   |
| 0.040            | 4.44                               | +3.78          | 4.62 | +1.63          | 4.99 | -2.17          | 5.16 | -3.21          | 4.80              |
|                  | 4.46                               | +3.29          | 4.64 | +1.85          | 5.01 | -2.02          | 5.19 | -3.63          |                   |
| Average.....     |                                    |                |      |                |      |                |      |                | 4.80              |

\* In the second experiment with 0.010  $M$  acetate the gelatin concentration was only 0.01 per cent instead of 0.1 per cent.

the previous sections for the isoelectric or isoionic point of gelatin in solution.

The cause of this difference is not given by the present data. One might speculate that there is an effect of salt on the isoelectric point, tending to lower its pH, which does not vanish even at 0.001 M, or that possibly the collodion selectively adsorbs more of the heat coagulable

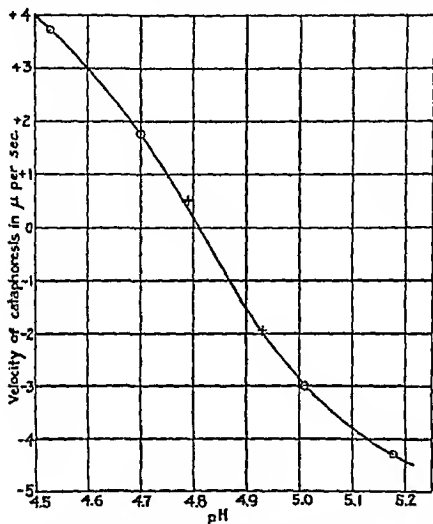


FIG. 3. Velocity of cataphoresis of collodion particles in 0.1 per cent gelatin in acetate buffers as a function of pH. The isoelectric point is given by the intersection of the curve with the line of zero velocity. This curve shows the results with a buffer containing 0.001 M sodium acetate, the intersection being at pH 4.81 in this case (Table II).

protein in the gelatin, which has been shown by Sheppard, Hudson, and Houck (1931) to have an isoelectric point in the vicinity of pH 4.0.

It may be noted that none of the above methods gives any indication of the existence of two isoelectric points at widely separated pH values. The inference of Johlin (1930) that gelatin apparently has two iso-

electric points at pH 4.68 and 5.26 was made from the intersections of certain viscosity curves. The more direct methods of the present work lend no support to such an assumption.

## VI

### SUMMARY AND CONCLUSIONS

Two samples of a standard gelatin were studied, both prepared according to published specifications and washed free from diffusible electrolytes. The isoelectric point of this material was determined in four ways.

1. The pH values of solutions of gelatin in water approached the limit  $4.86 \pm 0.01$  as the concentration of gelatin was increased.

2. The pH values of acetate buffers were unchanged by the addition of gelatin only at pH  $4.85 \pm 0.01$ . This gives the isoionic point of Sørensen, which is the isoelectric point with respect only to hydrogen and hydroxyl ions.

3. Gels of this gelatin made up in dilute HCl or NaOH, or in dilute acetate buffers, exhibited maximum turbidity at pH  $4.85 \pm 0.03$ .

4. Very dilute suspensions of collodion particles in 0.1 per cent gelatin solutions made up in acetate buffers showed zero velocity in cataphoresis experiments only at pH  $4.80 \pm 0.01$ .

No evidence was found for the assumption that gelatin has two isoelectric points at widely separated pH values.

It is concluded that the isoelectric point of this standard gelatin is not far from pH 4.85.

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# THE PHOTOTROPIC SENSITIVITY OF PHYCOMYCES AS RELATED TO WAVE-LENGTH

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## I

The action of light on the photosensitive meristematic region of the elongating sporangiophore of *Phycomyces* leads to a temporary acceleration of growth (the "light-growth" response). If the sporangiophore receives unequal illumination on opposite sides, phototropic bending typically occurs, directed toward the more intense source of light. In this case bending is due to unequal growth on opposite sides of the meristem, not to a flexion of the sporangiophore as a whole. These two modes of response—acceleration of growth and bending—have been shown to be parallel indicators of the state of photic excitability of the sporangiophore (Castle, 1929–30).

It is of interest to study the relative efficiencies of different spectral regions in exciting the photic system of *Phycomyces*, since the stimulating efficiency of each wave-length may be taken to represent the effective absorption of light at that wave-length,—and thus, with certain necessary reservations, the absorption spectrum of the photosensitive material presumed to initiate the chain of events leading to response. A detailed justification of such a procedure has been given by Hecht (1920–21), although others have mapped out "biological absorption spectra" for animal and plant systems on the same assumptions.

The reservations which must be made, however, in such reasoning are these: first, it is clearly impossible by this method to dissociate from the absorption of the sensitive substance that of any secondary, screening pigment associated with it. This consideration is of particular importance in the case of plants, where accessory pigments are common. Thus the sporangiophores of *Phycomyces* contain pigment,

the amount of which seems to vary with the intensity of illumination under which the fungus has been grown. The nature of this pigment and its rôle in determining the phototropic behavior of *Phycomyces* will be considered elsewhere.

In the second place, unequal refraction of different wave-lengths of light within the organ in question may introduce complications. Curved, photosensitive plant structures of small dimensions function somewhat as lenses. For example, the clear, cylindrical sporangiophore of *Phycomyces* concentrates light on the side more remote from the source of illumination. While the exact light paths are hard to visualize (Blaauw, 1914; Oehlkers, 1926), it is obvious that the light must pass through several concentric layers having different refractive indices. It is possible in this case that the different wave-lengths do not reach the same effective place of absorption.

The mapping out of "biological absorption spectra" does give definite, useful information as to the photic sensitivity of the system in question, although the result may not be at once identified with the absorption spectrum of the underlying sensitive substance. In this paper are presented the results of a study of the sensitivity of the sporangiophores of *Phycomyces* to different spectral regions.

## II

The method of experimentation depends on the fact that in a culture of *Phycomyces* placed between two equal sources of light opposed at  $180^\circ$ , the sensitive sporangiophores either (1) continue to grow upward at right angles to a line connecting the two sources (a state of continuous phototropic "indifference"), or (2) are oriented in approximately equal numbers toward each source—see Fig. 1. If the illuminations from the two sources are unequal, a relatively greater number of sporangiophores bend toward the more highly illuminated side.

This method of so-called "antagonistic" illumination has been used previously in work with the phototropic responses of sessile plants: by Massart (1888) to determine the discrimination of intensity by *Phycomyces nitens*; recently by Bergann (1930) to study the sensitivity of *Avena* coleoptiles to different spectral regions.

In the present experiments, the inclination of equal numbers of sporangiophores toward each source of light was found to be the most satisfactory end-point by which to judge equal phototropic effects.

The fact that the so-called "resultant law" does not hold rigorously for all individual sporangiophores may be due (1) to asymmetry of the sporangiophore, or (2) to initial chance orientation of individuals in the direction of each light, these sporangiophores then being held effectively oriented by one light alone. The latter is the most probable explanation of the "non-resultant" orientation of *Pilobolus* sporangiophores toward one or the other of two sources of light observed

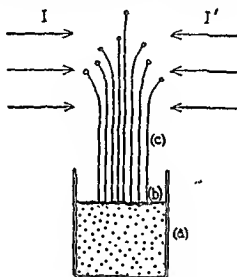


FIG. 1. Diagram of a culture of *Phycomyces* sporangiophores oriented between two equal sources of illumination ( $I = I'$ ). Equal numbers of individuals are shown bent in each direction, and in the middle the characteristic "indifferent" class. In a culture as actually used, the sporangiophores are arranged in a row at right angles to the plane of the paper, to avoid shading one another. The symmetrical arrangement is illustrative only. (a), culture vessel and medium; (b), flat, vegetative mycelium; (c), sporangiophores oriented by two equal, opposed illuminations.

by Allen and Jolivet (1914). In this respect it is significant that the young sporangiophores of *Pilobolus* placed between two sources of light obey the "resultant law" (Pringsheim and Czurda, 1927). At this time the sporangium has not been formed, and the path of light in the sensitive meristem is relatively simple, in contrast to the complex optical situation following sporangium-formation (Van der Wey, 1929).

The general procedure was to equate the phototropic effects of different spectral regions by means of cultures of elongating, sensitive sporangiophores placed in beams of light opposed at  $180^\circ$ . The rela-

tive intensities of the two beams were then adjusted until equal numbers of sporangiophores bent toward each source of light. At this point of equal phototropic effect, the efficiency of each spectral region was taken as inversely proportional to its relative energy content.

Opposed beams of light of equal intensity and identical spectral composition were obtained from a single source by mirrors placed at two points of an isosceles

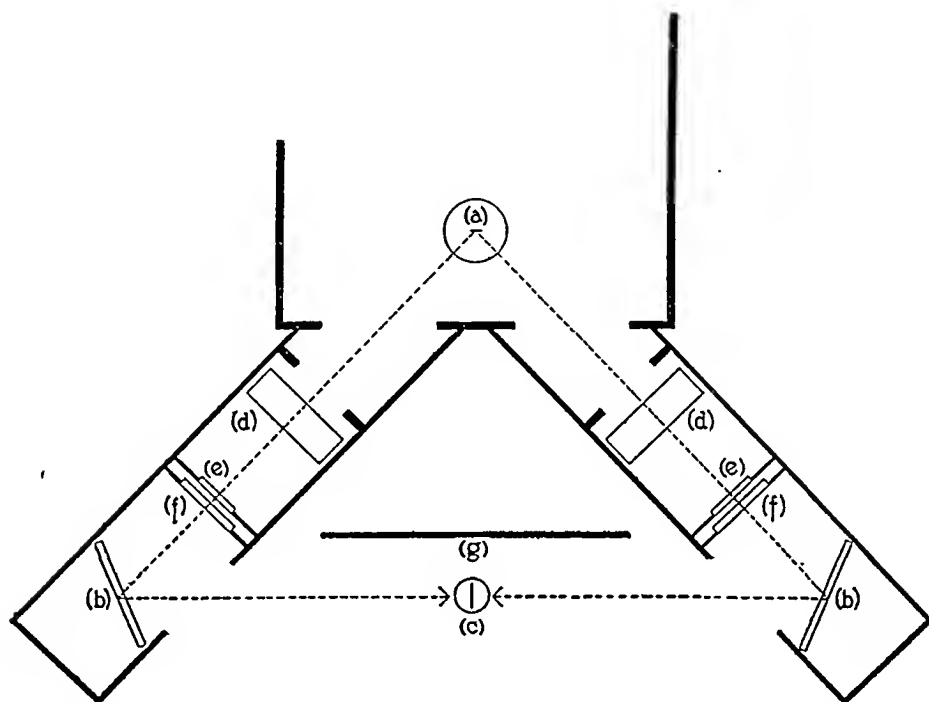


FIG. 2. Plan of apparatus with covers removed from lamp housing and beam housings (heavy lines). Dotted lines represent the light-paths coming from the 400 watt projection bulb (a), passing various filters (d), (e), (f), reflected by the mirrors (b) and falling on the culture of *Phycomyces* (c) from opposite sides. (d) cells containing  $\text{CuCl}_2$  solution; (e), neutral intensity filters; (f), Wratten "monochromatic" filters; (g), screen to shield cultures on the optical bench from stray heat radiation from lamp housing.

triangle (see Fig. 2). At the middle of the third side of this triangle the opposed illuminations were equal; at other points the intensities were unequal, each being found to vary inversely with the square of its distance from the lamp, which therefore approximated a point source. The relative intensities falling on opposite sides of a culture could thus be varied by changing the position of the culture

between the mirrors, or by the interposition of neutral intensity filters (Fig. 2, c) in the beams before reflection. For this latter purpose Eastman neutral filters were used. Special photometric calibration of these filters is essential, since the manufacturer's transmission values were found to deviate by as much as 10 per cent from the true values.

In order to secure relatively monochromatic light of high intensity, a 400 watt, 115 volt, plane-filament projection lamp was used as the source of illumination, in conjunction with Wratten "monochromatic" filters interposed in the two lateral beams (Fig. 2, f). This method of obtaining fairly monochromatic light has been

TABLE I

Relative spectral energy distribution of 400 watt, 115 volt projection lamp, and percentage transmission of glass cell containing a layer 3.3 cm. thick of 0.065 molar  $\text{CuCl}_2 \cdot 2\text{H}_2\text{O}$ .

| Wave-length | Relative energy<br>of source, color<br>temp 3000°K. | Transmission of<br>$\text{CuCl}_2$ screen | Wave-length | Relative energy<br>of source, color<br>temp. 3000°K. | Transmission of<br>$\text{CuCl}_2$ screen |
|-------------|---|---|-------------|--|---|
| ( $m\mu$ )  |   | (per cent)                                | ( $m\mu$ )  |  | (per cent)                                |
| 350         | 0.062   | —   | 490         | 0.614  | 89.5                                      |
| 360         | 0.084   | —   | 500         | 0.673  | 88.8                                      |
| 370         | 0.105   | —   | 510         | 0.738  | 88.0                                      |
| 380         | 0.130   | —   | 520         | 0.804  | 87.0                                      |
| 390         | 0.157   | —   | 530         | 0.870  | 85.8                                      |
| 400         | 0.187   | 66.0                                      | 540         | 0.934  | 84.0                                      |
| 410         | 0.223   | 72.5                                      | 550         | 1.000  | 82.3                                      |
| 420         | 0.262   | 76.0                                      | 560         | 1.068  | 79.9                                      |
| 430         | 0.301   | 82.5                                      | 570         | 1.145  | 76.0                                      |
| 440         | 0.344   | 85.5                                      | 580         | 1.203  | 71.5                                      |
| 450         | 0.392   | 87.3                                      | 590         | 1.268  | 66.5                                      |
| 460         | 0.443   | 88.7                                      | 600         | 1.334  | 60.0                                      |
| 470         | 0.495   | 89.5                                      | 610         | 1.400  | 53.8                                      |
| 480         | 0.554   | 89.8                                      | 620         | 1.465  | 47.5                                      |

described in detail by Hecht (1920-21), Bachmann (1929), and others. Infra-red radiation was screened from each beam by a glass cell containing a solution 3.3 cm. thick of 0.065 molar  $\text{CuCl}_2 \cdot 2\text{H}_2\text{O}$ . The percentage transmission factors of this filter were determined spectrophotometrically throughout the visible spectrum and used in the subsequent computations.

The relative energy content of each "monochromatic" beam was then calculated as follows: (1) the energy of the source at each wave-length was taken from a calibration of a similar 400 watt, 115 volt projection lamp having a color temperature of 3000°K, the relative energies being calculated by the use of Wien's equation (Table I) (see Forsythe, 1919 Report of Standards Committee on Py-

rometry, 1920). (2) The energy of the lamp at each 10  $m\mu$  interval was then multiplied in turn by the transmission factor for that wave-length of each of the filters interposed in the beam of light, *viz.*: the transmission factors of the copper chloride screen (Table I), of the neutral intensity filter (if used), (Table III), and of the particular Wratten filter interposed (Wratten Light Filters, Rochester, 1922). (3) The resulting values of transmitted radiation in 10  $m\mu$  steps were plotted on a large scale against wave-length, and the total areas under the curves measured. These areas of total energy transmission were taken as the relative intensities of radiation of the source in the spectral region transmitted. (4) The intensity of a beam at a particular point was calculated by the inverse square law.

Loss of intensity through reflection by the mirrors, which were cut from the same piece, was assumed to be uniform as regards wave-length, and was not corrected for except in the case of the Wratten ultra-violet filter No. 18. Here the path of light through the glass of the mirror totalled 13 mm., a thickness of crown glass

TABLE II

Transmissions calculated from data of Gibson, Tyndall, and McNicholas (1920) for 13 mm. of "nearly neutral" crown glass.

| Wave-length | Percentage transmission |
|-------------|-------------------------|
| ( $m\mu$ )  |                         |
| 350         | 33.0                    |
| 360         | 57.0                    |
| 370         | 63.0                    |
| 380         | 73.0                    |
| 390         | 75.0                    |

sufficient to absorb effectively all ultra-violet below 350  $m\mu$ . The transmissions calculated for this thickness of glass according to the figures of Gibson, Tyndall, and McNicholas (1920) are given in Table II. The energy of the source at each wave-length was multiplied by these factors in obtaining the total energy transmitted by Filter No. 18. Further, in order to get as much of the near ultra-violet as possible, the strongly absorbing copper chloride screen was not used in conjunction with this filter. Absorption of the near ultra-violet by the glass wall of the projection lamp was undetermined, and not corrected for, although the allowance which may be made for this in the final result will be pointed out shortly.

The apparatus comprising lamp and housing, beam housings, filters, mirrors, and optical bench on which the cultures were placed (see Fig. 2) was set up horizontally on a table in a dark room which maintained a relatively constant temperature (18.9 — 20.7°C.) during the duration of all the experiments. Pure cultures of *Phycomyces blakesleeanus* Burgeff ("+" strain) were grown on wet, sterilized flour paste in small glass vials of 2.5 cm. diameter. Prior to experimen-

tation, sporangiophores were developed by keeping the cultures in a warm, moist chamber illuminated from above by light of constant intensity. At the end of some hours there was obtained a good growth of young, vertical sporangiophores 2-5 cm. tall and equally light-adapted. In order to obviate shading within a culture, and also to reduce the loss of moisture on the optical bench, each glass culture vessel was provided with a metal cover having a slit 1-1.5 mm. wide cut in it. This device allowed a limited number of sporangiophores to grow up through the slit in practically a single row, precluding shading when the slit was placed across the axis of the beams of light.

Comparable cultures of equally light-sensitive sporangiophores were placed singly between the mirrors and allowed to remain undisturbed except for the bilateral illumination, for a minimum of 4 hours. At the end of this time the number of sporangiophores bent toward each source and those remaining "indifferent" were recorded. When the general zone of phototropic balance was determined, it was systematically explored in a series of separate experiments by placing cultures at successive small intervals along it. Thus for each pair of opposed monochromatic beams there was obtained a relation between the position on the optical bench and the ratio of the sporangiophores in a culture bent in one direction to those bent in the other. These relations were plotted, and graphically interpolated to find for each the locus of equal phototropic bending. At some distance from the equalization-point the plots are frequently irregular, but are surprisingly smooth near the point of equal phototropic effect. The equalization point was determined to within 2 cm. in the case of the least phototropically effective spectral regions where orientation is least precise. Assuming the measured end-point to be near the middle of the optical bench, a maximum error of 2 cm. in the setting would produce at most an intensity change of 3 to 4 per cent in the calculated intensity of each beam.

### III

Four separate experiments were performed in equating the phototropic effects of different spectral regions. These are detailed in Table III. Since it was not convenient to equate the spectral regions in turn against one constant one, the four series have to be brought to the same common denominator of intensity by simple proportionality.



The results of this calculation are given in Table IV, graphically in Fig. 3.

It is clear from Fig. 3 that the short wave-lengths of the visible spectrum are the most effective in exciting the light-sensitive system of *Phycomyces*, with a maximum probably in the extreme visible between 400 and 430  $m\mu$ . The sensitivity in the near ultra-violet is striking, and should be even greater than the calculated value since absorption by the glass wall of the projection lamp is not corrected for. Toward the longer wave-lengths there is a sharp drop in sensitivity to a value of almost nothing at 580  $m\mu$ .

TABLE III

Distance necessary for phototropic balance for each pair of opposed "monochromatic" beams of light obtained by the use of Wratten filters.

| Series | Left beam          |   |  | Right beam         |   |  |
|--------|--------------------|---|--|--------------------|---|--|
|        | Wratten filter no. | Percentage transmission of neutral filter (if used) | Critical distance from culture to lamp for phototropic balance | Wratten filter no. | Percentage transmission of neutral filter (if used) | Critical distance from culture to lamp for phototropic balance |
|        |                    |   | (cm.)  |                    |   | (cm.)  |
| 1      | 76                 | —   | 38.5   | 75                 | —   | 32.7   |
| 2      | 74                 | —   | 41.0   | 75                 | 0.38  | 30.2   |
| 3      | 74                 | 50.8  | 40.0   | 73                 | —   | 31.2   |
| 4      | 76                 | 23.6  | 39.8   | 18*                | —   | 51.2   |

\* Copper chloride screen not used in right beam of this experiment.

Blaauw (1909) determined the relative efficiencies of different wave-lengths of light in causing just perceptible bending of the sporangio-phores of *Phycomyces nitens*. His results are also plotted in Fig. 3, and show a maximum at 495  $m\mu$  with decreasing sensitivity in the blue. The displacement of the maximum some 80  $m\mu$  toward the blue end of the spectrum in the present experiments may reflect a specific difference between the photosensitive systems of the two species of *Phycomyces*, or distinct states of sensitivity due to the different circumstances of experimentation. Blaauw worked with relatively brief exposures (between 16 and 192 sec.) to a narrow region of a dispersed spectrum of low intensity, whereas in the present experi-

TABLE IV

Relative effectiveness of different spectral regions in stimulating *Phycomyces*.

| Written filter no. | Wave-lengths between which are contained 1/2 of energy transmitted by filter as used in the experiment | Relative energy necessary to produce same phototropic effect | Relative effectiveness in stimulation = reciprocal of relative energy necessary to produce the same phototropic effect; No. 76 = 100 |
|--------------------|--|--|--|
|                    | (m $\mu$ )   |  |  |
| 76                 | 429-448  | 0.0124   | 100.0  |
| 18                 | 356-376  | 0.0248   | 50.0   |
| 75                 | 477-495  | 0.0474   | 26.2   |
| 74                 | 521-535  | 7.82   | 1.6  |
| 73                 | 564-580  | 10.3   | 0.12   |

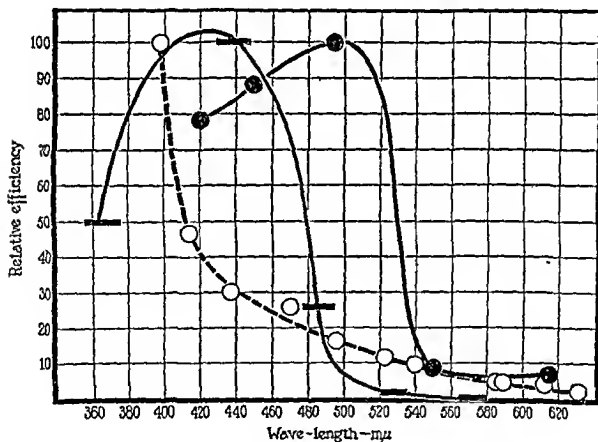


FIG. 3. Relative efficiency in stimulation of different wave-lengths. Solid bars, *Phycomyces blakesleanus*, data from Table IV. The length of each bar represents the range within which one-half of the energy transmitted by each combination of filters is contained. Solid circles, *Phycomyces nilens*, data from Blaauw (1909). Open circles, *Pilobolus*, calculated from data of Parr (1918). The most effective point in stimulation of each series of measurements is arbitrarily put at 100.

ments wider spectral regions of much greater intensity were allowed to act for 4 hours. More pigment initially present in Blaauw's sporangiophores would cause relatively less effective absorption in the blue, assuming a strong screening action in that region by the pigment. Preliminary experiments indeed show this to be the region of greatest absorption of the pigments extracted from the sporangiophores by acetone or other fat solvents.

#### IV

Since the work of Blaauw (1909) little of quantitative significance has been learned about the photosensitivity of fungi as related to wave-length. An exception is the study by Parr (1918) of the phototropic responses of *Pilobolus*. If a proper measure of sensitivity is chosen, namely, the reciprocal of the amount of energy necessary at each wave-length to produce the same phototropic effect, the data of Parr can be plotted in the form of an absorption spectrum (see Fig. 3). In order to compute the amount of energy needed, the validity of the Bunsen-Roscoe law must be assumed for exposure times of 50-80 minutes duration. While there are objections to this assumption, the procedure is the only way of properly expressing the results of the experiments, which the author failed to do. Thus interpreted, the data of Parr show that the young sporangiophores respond to light over the whole visible range of wave-lengths, with regularly increasing sensitivity down to 398  $m\mu$ , where the experiments ceased. No maximum of sensitivity was found within this range.

Numerous studies relating wave-length and sensitivity have been made with other sessile plants such as the coleoptiles of *Avena* (Blaauw, 1909; Koningsberger, 1922; Sonne, 1929; Bachmann and Bergann, 1930). With certain differences, all agree in showing the greatest sensitivity to be within the region 425-475  $m\mu$ , although there is no reason to suppose that the plants used in the different experiments were equally light- or dark-adapted, or were similarly etiolated. Studies of the photosensitivity of free-swimming, green flagellates show their respective maxima to be even more displaced toward the red, *c.g.*, at 483  $m\mu$  for *Euglena* and *Gonium* (Mast, 1917); at 494  $m\mu$  for *Volvox* (Laurens and Hooker, 1920).

It is clear that the photosensitive systems of fungi about which

we have significant information absorb more effectively in the short wave-lengths of light than do those of chlorophyll-containing organisms or of animals. It is therefore of particular interest to know what rôle, if any, in the photic responses of such fungi is played by their pigments which absorb the short wave-lengths.

## SUMMARY

Under the circumstances of experimentation described, the sporangio-phores of *Phycomyces* are found to be most sensitive to stimulation by light in the violet between 400 and 430  $m\mu$ . Toward the red, sensitivity falls to nearly zero near 580  $m\mu$ , while in the near ultra-violet around 370  $m\mu$ , sensitivity is still high. The previous experiments of Blaauw had placed the point of greatest sensitivity some 80  $m\mu$  nearer the red end of the spectrum. Because of the known presence in the sporangiophores of *Phycomyces* of "accessory" pigments, care must be taken in identifying such results with the absorption spectrum of the photosensitive substance.

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## CRYSTALLINE PEPSIN

### III. PREPARATION OF ACTIVE CRYSTALLINE PEPSIN FROM INACTIVE DENATURED PEPSIN

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#### INTRODUCTION

The results (1) of the experiments with crystalline pepsin isolated from crude pepsin preparations indicated that the material is a pure substance and that the proteolytic activity is a property of the protein molecule itself and is not due to the presence of a separate non-protein impurity. No indication of the presence of a more highly active non-protein molecule was obtained in the solubility measurements, inactivation experiments, or measurements of the rate of diffusion. In a sense however, this evidence is all negative in that it merely fails to show the presence of a more active molecule. It was shown, for instance, that the loss in activity in alkaline solution was quantitatively paralleled by the production of insoluble denatured protein. It could be objected, however, that the enzyme was liberated from the protein when the latter became denatured and that the enzyme itself was too unstable to exist alone. If it could be shown, however, that reversal of denaturation of the protein was accompanied by reactivation of the enzyme and that the native protein obtained in this way had the same activity as the original protein, strong proof would be furnished for the assumption that the activity was really a property of the protein molecule. In order to account for this result on the hypothesis that the activity was due to the presence of a small amount of a more highly active substance it would be necessary to suppose that the conditions for transforming the denatured protein into the original pro-

tein and for reactivating the enzyme were nearly identical—an assumption which is highly improbable.

It was found by Pawlow and Parastschuk (2) that pepsin solutions which had been inactivated by alkali recovered some of their activity when allowed to stand in nearly neutral solution. It was shown by the writer (1) that this alkali-inactivation of the enzyme is quantitatively paralleled by the formation of denatured protein as determined by the production of protein insoluble near the isoelectric point. The procedure used by Pawlow and Parastschuk for reactivating the enzyme was identical with that found by Anson and Mirsky (3) for the reversal of denaturation of proteins. This suggests that the reactivation of pepsin is due to the reversal of coagulation of the pepsin protein. If this explanation is correct it should be possible to isolate active pepsin protein from such reactivated solutions. The experiments reported in this paper show that this can be done.

### *Experimental Results*

Pawlow and Parastschuk's results were repeated more carefully by Tichomirow (4) who apparently could recover about 10 per cent of the original activity using dog gastric juice. Tichomirow inactivated the enzyme by adding strong alkali or sodium carbonate and then titrated the solution to slightly less than neutrality as shown by litmus. When the solution was allowed to stand under these conditions a gradual increase in activity was found. Exact repetition of these experiments is difficult since at the time of Tichomirow's work pH measurements were not known. The exact degree of neutralization could not be duplicated with certainty from the data given. It is evident from Tichomirow's work, however, that the reaction is dependent upon the pH at which inactivation takes place, the pH and temperature at which reactivation occurs, and the length of time for which the solutions are allowed to stand under these conditions. In addition, the salt concentration and enzyme concentration are other possible variables.

It has been shown by Goulding, Borsook and Wasteneys (5) that two reactions are involved in the inactivation of pepsin by alkali. The first reaction is instantaneous and its *extent* is determined by the pH to which the solution is brought, while the *rate* of the second reaction

is determined by the pH. When the percentage inactivation caused by the instantaneous reaction is plotted against the pH a titration curve is obtained with a pK of about 6.9. This result has been confirmed with crystalline pepsin solutions, and it was shown in addition that the percentage inactivation was exactly parallel with the percentage of the protein denatured.

Preliminary experiments showed that a solution of crystalline pepsin which has been inactivated by titrating with NaOH to pH 10 or 11 (pink to Nile blue) becomes slightly active again when allowed to stand

TABLE I

*Effect of pH of Solution during Reactivation*

100 ml. crystalline pepsin solution titrated to pH 10.5 with NaOH at 22°C. for 10 min. Titrated to pH 6.0 + HCl and diluted 1/8 with M/100 citrate buffer of different pH. Allowed to stand at 22°C. for 18 hrs. and activity determined.

| pH of solution after diluting with citrate buffer.....                     | 5.0  | 5.3  | 5.6  | 5.9  | 6.2  |
|--|------|------|------|------|------|
| [PU] <sub>ml.</sub> × 10 <sup>3</sup> of original solution {gelatin V..... | 1.0  | 1.0  | 1.0  | 1.0  | 1.0  |
| {casein F.....   | 100  | 100  | 100  | 100  | 100  |
| After inactivation {gelatin V.....   | 0    | 0    | 0    | 0    | 0    |
| {casein F.....   | 0    | 0    | 0    | 0    | 0    |
| After reactivation {gelatin V.....   | 0.02 | 0.07 | 0.13 | 0.16 | 0.12 |
| {casein F.....   | 0.10 | 0.40 | 0.40 | 0.20 | 0.10 |
| Per cent of original, reactivated {gelatin V.....                          | 2.0  | 7.0  | 13.0 | 16.0 | 12.0 |
| {casein F.....   | 0.10 | 0.40 | 0.40 | 0.20 | 0.10 |

after partial neutralization. The yields, however, were less than 1 per cent instead of 10 per cent as described by Tichomirow. The conditions were therefore varied systematically in an attempt to increase the yield. The first condition studied was the effect of varying the pH to which the solution was brought after complete inactivation by alkali. It was found that the maximum reactivation was obtained at about pH 5.4 for the casein-hydrolyzing activity, while the gelatin liquefying activity was recovered more completely



in slightly more alkaline solution.<sup>1</sup> The results of one of these experiments are shown in Table I.

In the experiment just described inactivation was caused by strong alkali and was therefore probably due almost entirely to the instantaneous reaction rather than to the slower secondary reaction. It seemed possible that better yields might be obtained if the inactivation were brought about by the slow reaction. However, experiment showed that this was not the case but that on the contrary no reactivation could be obtained in solutions which had been allowed to inacti-

TABLE II

*Effect of pH during Inactivation on Reactivation*

Series of 10 ml. crystalline pepsin solutions titrated to pH noted. Kept at 20°C. until more than 90 per cent of activity had been lost. Titrated to pH 5.4, kept at 22°C., and activity determined after 24 to 48 hrs.

| pH at which inactivation occurred.....                                     | 7.0  | 8.0 | 8.5  |
|--|------|-----|------|
| [PU] <sub>ml.</sub> × 10 <sup>3</sup> of original solution {gelatin V..... | 4.0  | 4.0 | 4.0  |
| {casein F.....   | 400  | 400 | 400  |
| After inactivation {gelatin V.....   | 0.4  | 0.5 | 0.1  |
| {casein F.....   | 0.10 | 0   | 0    |
| After reactivation {gelatin V.....   | 0.4  | 0.6 | 0.2  |
| {casein F.....   | 0.8  | 0.4 | 0.35 |
| Per cent reactivated {gelatin V.....                                       | 0    | 2.5 | 2.5  |
| {casein F.....   | 0    | 0.1 | 0.1  |

vate slowly at lower pH's. The results of this experiment are shown in Table II.

*Effect of the Time of Standing after Neutralization.*—When the solution is brought back to pH 5.4 and tested at once for activity it is found to be completely inactive. The activity slowly increases for

<sup>1</sup> The variation in the activity as measured by the liquefaction of gelatin or the hydrolysis of casein after alkali inactivation is due to the fact that the crude pepsin contains a gelatin liquefying enzyme which can only be removed by repeated crystallization. This gelatin splitting enzyme is more resistant to alkali than the pure pepsin. This point will be discussed more fully in a subsequent paper.

24 to 48 hours and then decreases so that apparently there are again two reactions, one of which leads to the formation of active enzymes while on the other hand, the active material is being slowly destroyed. The amount of active material actually recovered then is the result of these two reactions. The results of such an experiment are shown in Table III.

*Effect of Concentration on Percentage of Reactivation.*—The work of Anson and Mirsky on the reversal of denaturation of proteins indicates that the reaction is dependent somewhat upon the solubility of the denatured protein. Conditions which increase the solubility of the denatured protein are favorable for reversal while conditions which

TABLE III

*Effect of Time at pH 5.4 on Reactivation*

0.05 per cent pepsin solution titrated to pH 10.5 with NaOH at 22°C. for 10 min. Titrated to pH 5.4 with N/2 HCl. Activity determined after various time intervals at 22°C.

| Hrs. at 22°C. | [FU] <sub>ml.</sub> × 10 <sup>3</sup> |           |
|---------------|---------------------------------------|-----------|
|               | Gelatin V.                            | Casein F. |
| 0             | 0                                     | 0         |
| 2             |                                       | 0 02      |
| 18            | 0 03                                  | 0 20      |
| 42            | 0 03                                  | 0 21      |
| 65            | 0 04                                  | 0 25      |
| 90            | 0 045                                 | 0 12      |
| 114           | 0 040                                 | 0 10      |

cause precipitation of the denatured protein are unfavorable. It seemed possible therefore that better yields would be obtained with more dilute solutions and this was found to be the case. There is even some indication that the concentration of active material recovered is nearly constant so that the percentage of activity recovered increases with the dilution. Table IV shows the results of the experiment in which the solution was diluted to various extents after being titrated to pH 5.4. Practically, however, it is not possible to work with solutions containing much less than 1 mg. of nitrogen per ml. owing to the very large volume.

A number of other experiments were done in an attempt to increase the percentage yield but without success. It is quite possible that the difference between these results and those of Tichimorow are due to the difference between dog pepsin and pepsin from pigs, since Anson and Mirsky have found that there is very considerable difference in the ease of reversal of hemoglobin from different animals.

*Tests for the Presence of Inhibiting Substances.*—The possibility exists that the loss in activity is due not to destruction of the enzyme but to the formation of an inhibiting substance which depresses the activity of the enzyme and which disappears on standing at pH 5.4 and thus liberates the active enzyme. This possibility can be rendered remote by testing the effect of the inactivated solution upon the ac-

TABLE IV

*Effect of Concentration on Reactivation*

Solution of crystalline pepsin titrated to pH 10.5 with  $N/2$  NaOH at 20°C. for 10 min. Titrated to pH 5.4 with HCl, diluted with water, and kept at 22°C. for 18 hrs.

| Mg. N/ml.....  | 8   | 4    | 2    | 1   | 0.5 |
|--|-----|------|------|-----|-----|
| [PU] <sub>ml.</sub> $\times 10^3$ (casein F.) — original solution..... | 800 | 400  | 200  | 100 | 50  |
| Immediately after titrating to pH 5.4.....                             | 0   | 0    | 0    | 0   | 0   |
| After 24 hrs. at pH 5.4..  | 1   | 1    | 0.7  | 0.5 | 0.4 |
| Per cent of activity recovered.....                                    | 0.1 | 0.25 | 0.35 | 0.5 | 0.8 |

tivity of normal pepsin solutions. If an inhibiting substance were present it would be expected that the activity of a known amount of active enzyme when added to this inactivated solution would be decreased since a large excess of inhibiting agent is usually necessary to completely inactivate the enzyme. No evidence for the presence of inhibiting substances could be found since active pepsin solutions diluted with a large excess of inactive solution show the same activity as when diluted with water. This result is shown in Table V.

*Tests for Completeness of Inactivation.*—As shown in the previous paper the denatured protein itself is rapidly digested by the enzyme, and this furnishes a very sensitive test for the presence of any active

pepsin in the solution after inactivation by alkali. The inactivated solution when brought to pH 2.5 to 3 forms a heavy precipitate of denatured protein. Under these conditions this protein is rapidly digested if any active enzyme is present. It was found that when the inactivated solution was titrated to pH 2.5 no increase in soluble nitrogen could be detected after 24 or 48 hours at 37°C. The addition of 4 per cent of the same solution which has been allowed to reactivate at pH 5.4 caused a marked increase in the amount of soluble nitrogen under these conditions (Table V). It may therefore be stated with

TABLE V

*Activity Determination on Inactivated Pepsin Solution*

6 per cent solution of crystalline pepsin titrated to pH 11.0 with  $N/2$  NaOH

|  | [PU] <sub>ml.</sub> × 10 <sup>4</sup> |           |
|--|---------------------------------------|-----------|
|  | Gelatin V.                            | Casein F. |
| 50 ml. titrated to pH 5 rapidly, slightly cloudy solution = a..... | 0                                     | 0         |
| 100 ml. titrated to pH 3 rapidly, precipitate forms = b....        | 0                                     | 0         |
| 25 ml. titrated to pH 5.4 and kept at 22°C. for 24 hrs. = c.....   | 0.05                                  | 0.5       |
| Active pepsin solution diluted 1/100 with H <sub>2</sub> O. ....   | 1.5                                   | 150       |
| with Solution a .....  | 1.6                                   | 165       |

0, 1 and 2 ml. Solution c added to 3 portions of 25 ml. of Solution b and nitrogen determined per ml. filtrate after 0 and 24 hrs. at 37°C.

| ml. reactivated Solution c. ....              | 0    | 1    | 2    |
|---|------|------|------|
| Mg. N/ml. filtrate after 0 hrs. at 37°C. .... | 0.10 | 0.12 | 0.15 |
| after 24 hrs. at 37°C. ....                   | 0.10 | 0.20 | 0.25 |

considerable certainty that not more than 2 per cent of the activity present in the reactivated solution was present after inactivation due to incomplete destruction of the original activity. In this experiment also no filtration or separation was made so that complicating factors such as adsorption of the active material by the precipitate, etc. seemed to be ruled out.

It had been found before that a mixture of active and inactivated pepsin could be separated by precipitating the inactive and denatured protein with a mixture of sulfuric acid and sodium sulfate. In the:

reactivated solutions obtained in the present work, however, it was found that the very small amount of active material present was carried down nearly completely by the denatured protein under these conditions, so that a new method of separation had to be worked out. After a number of failures it was found that most of the denatured protein could be precipitated from the solution by adding acid very slowly and carefully to the reactivated solution until it was just possible to filter off the precipitate formed. Under these conditions the active material is not carried down by the precipitate but remains in the filtrate. The addition of a slight excess of acid, however, causes all the active material to be retained by the precipitate. It seems probable that this result is due to the charge on the precipitate. The active pepsin is isoelectric at about pH 2.7 while the denatured protein is apparently isoelectric somewhere around 4.5, so that between these two pH's the two proteins are oppositely charged and precipitate each other, while if the reaction is adjusted so as to be on the alkaline side of the isoelectric points of both proteins they may be separated. The gelatin liquefying material is carried down with the precipitate even under these conditions so that this step serves to separate the reactivated pepsin from the "gelatinase" as well as from the denatured pepsin. The active pepsin may be recovered from this filtrate by adjusting to pH 3 and half saturating with magnesium sulfate. The precipitate obtained in this way contains about 2/3 of the total recovered activity but has a specific activity of only about 1/10 that of the original crystalline pepsin and evidently still contains large amounts of the denatured protein. Most of the latter may be removed by dialysis against 1/100 normal hydrochloric acid for 2 or 3 days. Under these conditions the denatured protein is partly digested by the active enzyme present. The resulting clear solution is precipitated with magnesium sulfate and the precipitate now has about half of its original specific activity. It still contains quite a large amount of some protein material which appears to be isoelectric at about pH 5 and from which the active enzyme can be separated only with great difficulty. Successive fractionation with magnesium sulfate at pH 5 gradually removes this inactive protein but no sharp separation could be made, and there is considerable loss in material at this point. Eventually, however, it was possible to obtain a small amount of a

TABLE VI

*Inactivation and Reactivation of Pepsin and Isolation of the Reactivated Enzyme*

| Procedure   | Fraction No. | Total [PU] |         | [PU] <sub>gm. N</sub> |         |
|---|--------------|------------|---------|-----------------------|---------|
|   |              | Gel. V.    | Cas. F. | Gel. V.               | Cas. F. |
| 100 gm. crystalline pepsin dissolved in 1 l. H <sub>2</sub> O   |              | 17         | 1700    | 1                     | 100     |
| Titrate to pH 10.5 (pink to Nile blue) + N/2 NaOH (800 ml.) at 22°C. for 10 min.  |              | <0.01      | <1      | 0                     | 0       |
| Dilute to 10 l.   |              |            |         |                       |         |
| Titrate to pH 5.4 with N/5 HCl, stirred in slowly so as to avoid local precipitation, at 22°C. for 24 hrs.  | 1            | 1.0        | 15      | 0.06                  | 1.0     |
| Titrate with N/5 HCl slowly until flocculent precipitate just forms (pH about 5.2).   |              |            |         |                       |         |
| Filter.   |              |            |         |                       |         |
| Filtrate  | 2            | 0.12       | 10      |                       | 4       |
| Filtrate 2. Titrate to pH 3.0 + H <sub>2</sub> SO <sub>4</sub> , MgSO <sub>4</sub> added till flocculent precipitate forms (about 350 gm. MgSO <sub>4</sub> per l.). Allowed to stand at 6°C. for 48 hrs. Supernatant siphoned off and precipitate concentrated by centrifuging. Centrifuged cake put in collodion sacs and dialyzed against 0.01 HCl at 22°C. for 48 to 60 hrs. Nearly clear solution. Centrifuge. Supernatant titrated to pH 5.4 with NaOH and MgSO <sub>4</sub> added until precipitate forms. |              |            |         |                       |         |
| Centrifuge, precipitate inactive.   |              |            |         |                       |         |
| Supernatant   | 3            | 0.012      | 1       | 0.6                   | 40      |
| Supernatant 3. MgSO <sub>4</sub> added until flocculent precipitate forms.  |              |            |         |                       |         |
| Centrifuge, precipitate weak activity. MgSO <sub>4</sub> added again to supernatant until precipitate forms. This process is continued until supernatant fluid has about proper specific activity per gm. N. The combined precipitates may be fractionated again and some active material recovered.  |              |            |         |                       |         |
| Combined Mother Liquors from MgSO <sub>4</sub> Precipitate.   | 4            | 0.006      | 0.5     | 0.9-10                | 90-100  |
| Solution 4. Titrate to pH 3.0 with H <sub>2</sub> SO <sub>4</sub> . Filter if cloudy and precipitate discarded.   |              |            |         |                       |         |
| Filtrate. Saturate with MgSO <sub>4</sub> and filter.   |              |            |         |                       |         |
| Precipitate   | 5            | 0.002      | 0.17    | 0.9-10                | 90-100  |
| Precipitate 5 from 3 preparations combined.   |              |            |         |                       |         |
| Dissolve at 40°C. with 0.2 to 0.3 ml. of water and 0.1 ml. N/10 Na acetate. Cool slowly. Typical hexagonal bipyramids in clusters form after 2 to 3 hrs.  |              |            |         |                       |         |

protein which had the general properties of the original crystalline pepsin. A summary of the method of isolation is shown in Table VI. About 2 kg. of the crystalline pepsin were used and the yields combined.

Control experiments like those shown in Table V were made on each preparation and showed that not more than 2 per cent of the activity could have been due to the presence of original enzyme which had not been inactivated. A small amount of this reactivated protein was crystallized under the same conditions as were used in crystallizing the original pepsin. It formed typical hexagonal bipyramids indistinguishable from those of the original pepsin. The activity as

TABLE VII  
*Comparison of Properties of Original and Reactivated Pepsin*

|                                   | [PU] <sub>gm. N</sub> |           |            | Mg. N to coagulate<br>5 ml. milk at 35°C.<br>in 2 hrs. | [α] <sub>22°C.</sub><br>pH 4.6 |
|-----------------------------------|-----------------------|-----------|------------|--|--------------------------------|
|                                   | Gelatin V.            | Casein F. | Edestin F. |  |                                |
| Original crystalline pepsin. .... | 1.1                   | 92        | 98         | <0.001>0.0005  | -70                            |
| Reactivated pepsin. ....          | 1.0                   | 95        | 100        | <0.001>0.0005  | -100 ± 10                      |
|                                   | 0.8                   | 98        | 110        |  |                                |

measured by the liquefaction of gelatin, the hydrolysis of casein, the hydrolysis of edestin and the coagulation of milk was the same as that of the original pepsin.<sup>2</sup> The optical rotation was measured on a very dilute solution and apparently showed a slightly different value from that of the original pepsin. The reading was somewhat uncertain, however, and it is doubtful whether this difference is significant. The

<sup>2</sup> It will be noted that the activity of the recovered as well as the original pepsin as determined by the digestion of edestin is only half of that originally reported for crystalline pepsin. This difference is apparently due to some difference in the edestin preparation since a different preparation was used in the present work. Unfortunately the edestin preparation used previously had been entirely used up but a sample of the original pepsin preparation was at hand both dry and in glycerin solution. This preparation now shows the same activity in all other respects as that found earlier but is apparently less active with the new edestin.

protein is denatured and the activity lost again in alkaline solution. The results of these determinations are shown in Table VII.

Attempts were made to compare the solubility of the reactivated and original pepsin but owing to lack of material these were not conclusive. It was found that the active material forms solid solutions with the protein which is carried along in the purification and which is apparently isoelectric at about pH 5. Addition of this protein to pure pepsin caused a marked decrease in solubility. The results with the rennet action confirmed Pawlow and Parastschuk's conclusion that the apparent separation of rennet from casein by alkaline activation is really due to the fact that the pepsin is reactivated under the conditions used for the coagulation of milk but not under the conditions used for the digestion of casein.

### *Experimental Methods*

The methods used were the same as those described in the study of crystalline pepsin. The pH determinations were made colorimetrically without correcting for any effect of the protein present and are only approximately correct.

### SUMMARY

1. Pepsin solutions which have been completely denatured and inactivated by adjusting to pH 10.5 recover some of their activity when titrated to about pH 5.4 and allowed to stand at 22°C. for 24 to 48 hours.
2. Control experiments show that this inactivation and reactivation are probably not due to the effect of any inhibiting substance.
3. A method of isolation of the reactivated material has been worked out.
4. The reactivated material recovered in this way is a protein with the same general solubility, the same crystalline form, and the same specific proteolytic activity as the original crystalline pepsin.
5. This furnishes additional proof that the proteolytic activity is a property of the protein molecule.



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# PROTEIN COAGULATION AND ITS REVERSAL

## SERUM ALBUMIN

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The reversal of the denaturation and coagulation of hemoglobin and globin has been described in previous papers (*cf.* review and literature in: Anson and Mirsky, 1931). This paper describes the preparation of crystalline, soluble, native protein from coagulated serum albumin and discusses the significance of the fact that this preparation offers so little difficulty.

### *Relation of the Ease of the Reversal of Denaturation to the Solubility of the Denatured Protein*

All denatured proteins are insoluble at their isoelectric points but soluble in acid or alkali. There are, however, considerable differences in the solubilities of different denatured proteins just as there are in the solubilities of different native proteins. Denatured hemoglobin is much less soluble than denatured serum albumin. In the absence of salts it is precipitated over a much wider range of hydrogen ion concentration around the isoelectric point than is denatured serum albumin and it is precipitated from acid solution by a concentration of salt which does not precipitate denatured serum albumin. Denatured egg albumin is even less soluble than denatured hemoglobin. Not only is it insoluble over a wide range of hydrogen ion concentration, but even when the denatured egg albumin appears to be dissolved by small amounts of acid or alkali there is aggregation or invisible precipitation of the protein (unpublished viscosity experiments).

Corresponding to these differences in solubility are differences in the ease of the reversal of denaturation. The denaturation of the relatively soluble serum albumin can be reversed with great ease; the denaturation of the less soluble hemoglobin can be reversed only by taking special precautions; while so far it has not been possible to reverse the denaturation of the very insoluble egg albumin at all.<sup>1</sup> Some 75 per cent of denatured serum albumin is converted into soluble native serum albumin on neutralization of an acid solution of the denatured protein. It makes no difference in the yield whether the protein is rapidly brought to the isoelectric point in one step, or first allowed to stand for half an hour in a solution just acid or alkaline enough to prevent precipitation. The same high yield is also obtained if the acid solution is one-tenth saturated with ammonium sulfate. In contrast, if an acid solution of denatured horse hemoglobin is rapidly brought to the isoelectric point, practically all the protein is precipitated. Reversal of denaturation is obtained, however, if the protein is first allowed to stand in a solution just acid or alkaline enough to prevent denaturation. Even with this procedure, no reversal is obtained and all the protein is precipitated, if the acid solution is one-tenth saturated with ammonium sulfate (Anson and Mirsky, 1925, 1929, 1930; Mirsky and Anson, 1929, 1930*a*). Finally, in the case of the very insoluble egg albumin, there is no reversal of denaturation or neutralization no matter how the neutralization is carried out.

Experimentally, then, there are two different results of neutralization of an acid solution of denatured protein: precipitation or aggregation of the insoluble denatured protein, and conversion of the insoluble protein into soluble native protein by the reversal of denaturation. In the three cases studied the more soluble the denatured protein is under the neutralization conditions, the easier and more complete is the reversal of denaturation.

The fact that in the cases of serum albumin, hemoglobin, and egg albumin, reversal of denaturation is more difficult the more insoluble the denatured protein does not mean that solubility is the only factor which determines to what extent reversal takes place or whether it can

<sup>1</sup>Recent experiments, which remain to be confirmed, indicate that the denaturation of egg albumin can under certain conditions be reversed.

take place at all. Unfortunately the theory of the mechanism of the reversal of denaturation (like the theory of the mechanism of denaturation itself) is as yet in an unsatisfactory state. It is not clear why reversal should result at all on neutralization, since native proteins are unstable at their isoelectric points.<sup>2</sup> For instance, isoelectric methemoglobin or egg albumin coagulates slowly on standing, and rapidly on being shaken with air or toluol globules. Nor is it clear why it should not be possible to reverse denaturation completely, at least on repetition of the reversal procedure. If the reversal procedure is carried out with denatured hemoglobin, 65 per cent of the protein is converted into a soluble form while 35 per cent remains insoluble. If the reversal procedure is repeated with the part that remained insoluble, this time much less than 65 per cent of the protein is made soluble. The solution of the non-reversed fraction does not behave the same as the solution of the original denatured hemoglobin.

A possible factor in causing this incompleteness of reversal is secondary irreversible change in the protein caused by the denaturation procedure but distinct from denaturation. In the case of hemoglobin there is not as yet any experimental evidence of such secondary change. It is known, however, that when egg albumin is heated there is, entirely apart from the formation of insoluble protein, a splitting off of ammonia (Sørensen and Sørensen, 1925). Particularly interesting experiments have been done with pepsin. Kühne (1877) discovered that pepsin is inactivated by alkali. Goulding, Borsook, and Wasteneys (1926) showed that there are two different kinds of inactivation by alkali, a rapid kind which does not go to completion except in strong alkali, but the *extent* of which depends on the pH; and a slow kind which always goes to completion, but the *rate* of which is proportional to the hydroxyl ion concentration. Northrop (1930; 1931), working for the first time with the pure pepsin protein, showed that both kinds of inactivation are accompanied by a formation of protein insoluble at the isoelectric point. The rapid inactivation and formation of insoluble protein, however, can be partially reversed by a procedure identical with that used for the reversal of denaturation, while by the

<sup>2</sup> It must be remembered in this connection that surface coagulation is most rapid at the isoelectric point. Under the conditions for reversal, it is much slower, indeed may not take place at all.

same procedure the slow kind of inactivation cannot be reversed at all. The simplest explanation of these results is that the rapid inactivation is simply denaturation of the protein, while the slow inactivation is a secondary irreversible change, perhaps a hydrolysis.

*Evidence That the Protein Has Been Denatured*

*Insolubility.*—To prove that denaturation has been reversed it must be proved that the protein has been denatured in the first place. The serum albumin used in the neutralization experiments is prepared by the addition to native serum albumin of acid acetone which is, in general, an effective denaturing agent. Usually one can demonstrate that protein treated with acid acetone is denatured by showing that the protein is completely precipitated when brought to the isoelectric point. The relatively soluble denatured serum albumin, however, seems to be converted into native protein by just this procedure. This difficulty may be avoided by neutralizing at a temperature at which reversal does not take place. If native serum albumin is heated in acid solution, the protein is denatured but is kept in solution by the acid. If the acid is neutralized while the solution is still hot, the protein is completely precipitated. This precipitate of insoluble and hence denatured serum albumin may be separated, dissolved, and converted, with a yield of 65 per cent, into protein soluble in half saturated ammonium sulfate.

*Effect of Heating and Trichloroacetic Acid on Yield.*—Were 75 per cent of the acid acetone serum albumin undenatured and were there no reversal on neutralization, then heating the acid acetone protein or precipitating it with trichloroacetic acid ought to result in further denaturation and hence in a lowering of the yield of soluble protein on neutralization. Actually heating and trichloroacetic acid have no effect on the yield, indicating that denaturation by acid acetone has been complete and that soluble protein is obtained by the reversal of denaturation.

*SH and S-S Groups.*—The high cystine content of serum albumin facilitates an entirely different and independent test for the completeness of denaturation and the reality of reversal. In general native proteins have few, if any, free sulfhydryl and disulfide groups, while denatured proteins have a number of such groups corresponding to

the total number of cystine and cysteine groups in the protein (Mirsky and Anson, 1930b; and unpublished experiments). Serum albumin treated with acid acetone or trichloroacetic acid has the number of free sulfhydryl and disulfide groups characteristic of a denatured protein, and when soluble serum albumin is produced by the reversal of denaturation there is a corresponding disappearance of these groups (unpublished experiments).

### *Historical*

The reversal of the denaturation of serum albumin was probably observed by Michaelis and Rona (1910). The experiments were not conclusive and there was some confusion in their interpretation, and so this investigation unfortunately never received the attention it deserved. Spiegel-Adolf (1926), however, showed definitely that if heat coagulated serum albumin is dissolved in alkali or acid and then electrodialed, soluble, heat coagulable protein is again obtained. No attempt was made to crystallize the protein. Spiegel-Adolf believed, in addition, that certain compounds of serum albumin with acid and alkali can be heated without the protein being denatured at all. Actually, one cannot tell from the type of experiment given in support of this conclusion whether serum albumin has been denatured or not.

### EXPERIMENTAL

*Preparation of Native Serum Albumin.*—Defibrinated horse blood is left in the cold until the corpuscles have settled. The serum is siphoned off and half saturated with ammonium sulfate which precipitates the globulins and the few corpuscles which remain suspended in the serum. To each liter of filtrate is added 20 gm. solid ammonium sulfate which results in an immediate and complete precipitation of the serum albumin in the amorphous form. Crystallization which is much slower and less complete than amorphous precipitation has the disadvantage that the protein may be fractionated and may in any case be changed on standing. The amorphous precipitate is filtered off and dialyzed in the cold against distilled water.

*Preparation of the Acid Acetone Powder.*—The preparation is the same as that of the acid acetone powder of globin (Anson and Mirsky, 1930). To a 5 per cent solution of serum albumin in 0.05 N HCl is added ten times its volume of acetone containing 2 cc. of 5 N HCl per liter. The resulting precipitate is filtered, washed with acetone, pressed as dry as possible, and then dried in the air. The dry albu-

min hydrochloride readily dissolves in water to give a clear solution. Most of the pigment present with the native serum albumin remains in the acid acetone when the protein is precipitated. The small amount of pigment which remains with the protein is more green and less yellow than the original pigment.

*Neutralization Experiments.*—If 0.1 N NaOH is gradually added to a 2 per cent solution of the acetone powder a point is reached at which a small precipitate is produced which increases with time. If 10 per cent more alkali is added the precipitate is redissolved. If 15 per cent less alkali is added no precipitate at all is formed.

The addition of an equal volume of ammonium sulfate to a solution which has been neutralized to the precipitation point causes further precipitation. Some 70 to 75 per cent of the protein, however, remains in the solution and has the properties of native serum albumin. The same result is obtained when the protein is allowed to stand half an hour in more acid or alkaline solution before being brought to the precipitation point and half saturated with ammonium sulfate. The table gives the results of such an experiment.

TABLE

| Ml. of 0.1 N NaOH added to 10 ml. of 2 per cent acid albumin | Per cent of protein not precipitated by half saturation with ammonium sulfate |
|--|---|
| 2.6 — precipitation point                                    | 74  |
| 0.8 × 2.6  | 77  |
| 0.9 × 2.6  | 73  |
| 1.1 × 2.6  | 68  |
| 1.2 × 2.6  | 78  |

*Estimation of Yield with the Phenol Reagent.*—To 1 ml. of filtrate from the solution half saturated with ammonium sulfate (in a Pyrex test tube, not a pointed centrifuge tube) is added 9 ml. of water, and after mixing, 2 ml. of 20 per cent trichloroacetic acid. The suspension is centrifuged and the supernatant solution poured off. The precipitate is dissolved in 2 ml. of 0.1 N NaOH and transferred with 23 ml. of water to a 50 ml. Erlenmeyer flask. 1 ml. of the phenol reagent (Folin and Ciocalteu, 1927) is added and then 1 ml. of 3 N NaOH. After 10 minutes the blue color developed is compared with the blue color developed from a known amount of acid acetone albumin similarly precipitated with trichloroacetic acid.

The estimation of proteins with the original phenol reagent of Folin and Denis (1912) as introduced by Wu (1922) does not yield colors proportional to the protein concentrations. Proportionality is obtained by the use of a higher concentration of the more soluble form of the phenol reagent described by Folin and Ciocalteu. Greenberg (1929) has made this same modification of Wu's procedure. The quantities he uses are somewhat different from ours and he adds the

phenol reagent after the alkali. In our experience, the procedure of Greenberg does not always yield perfectly clear solutions.

*Effect of Salt.*—If 1 ml. of saturated ammonium sulfate is added to the 10 ml. of the 2 per cent acid albumin, then on neutralization with 2.6 ml. of 0.1 N NaOH and half saturation with ammonium sulfate, 68 per cent of the protein remains in solution.

*Effect of Heating.*—The acid acetone solution is heated to 100°C. for 3 minutes. On neutralization a 70 per cent yield of soluble protein is obtained.

*Effect of Trichloroacetic Acid.*—To 10 ml. of 2 per cent acid albumin is added 20 ml. of water and 4.5 ml. of 20 per cent trichloroacetic acid. The precipitate is centrifuged, dissolved by the addition of a minimum amount of 0.1 N NaOH, and the solution after being made up to 12.6 ml. with water is half saturated with ammonium sulfate. The yield of soluble protein is 76 per cent.

In a similar experiment, native serum albumin is precipitated with 5 per cent trichloroacetic acid and the precipitate dissolved in an amount of alkali exactly equivalent to the trichloroacetic acid which remains with the protein. The yield of soluble protein is 74 per cent.

*Reversal of Heat Denaturation.*—A mixture of 10 ml. of 2 per cent dialyzed native serum albumin and 3 ml. of 0.1 N HCl is heated for 3 minutes at 100°C. and then while still hot neutralized with 2.5 ml. of 0.1 N NaOH. The resulting suspension is centrifuged. The clear supernatant liquid (which gives only a haze with trichloroacetic acid) is rejected and the precipitate is readily dissolved in 3 ml. of 0.1 N HCl plus enough water to give a final volume of 15 ml. 1 ml. is removed to provide a standard in estimating the yield. The 14 ml. are neutralized with 2.9 ml. of 0.1 N NaOH and half saturated with ammonium sulfate. 65 per cent of the protein is left in the solution.

*Crystallization of "Reversed" Serum Albumin.*—The serum albumin may be crystallized from the neutralized solution of 2 per cent albumin by adding to each 10 ml. of the solution half saturated with ammonium sulfate 2 to 2.5 ml. of saturated ammonium sulfate. It is better, however, to start with a more concentrated solution such as can be obtained by neutralizing a 5 per cent solution of acid albumin with 0.2 N NaOH. In this case 1.5 to 2 ml. of saturated ammonium sulfate per 10 ml. of half saturated suffices. The soluble serum albumin can be crystallized completely which indicates that half saturation with ammonium sulfate results in complete precipitation of denatured serum albumin even in the presence of a great excess of native serum albumin. The crystals of "reversed" serum albumin appear to have the same form as those of normal, native serum albumin.

## CONCLUSIONS

1. It is possible to prepare crystalline, soluble, heat-coagulable serum albumin from coagulated serum albumin.

2. In the cases so far studied, the more soluble a denatured protein, the more easily its denaturation can be reversed.



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# THE DEFORMABILITY AND THE WETTING PROPERTIES OF LEUCOCYTES AND ERYTHROCYTES

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A principal function of the leucocytes is phagocytosis, the removal by ingestion of foreign particles and the products of injury from the blood and tissue spaces. The process of phagocytosis involves the spreading of the leucocyte over the surface of a particle until the latter is completely enclosed. In the body, phagocytosis is enormously augmented by the deposit of serum proteins on the surface of the particle, and it is proving possible to define with some exactness the characteristics of the surface thus formed on the particle which produces a maximum tendency to phagocytosis (1). Concerning the spreading surface, the surface of the leucocyte, however, much less is known. The present study was undertaken principally with the object of gaining such information about the phagocytes and their surfaces as was possible by the interfacial technique; it was later broadened to include a study of lymphocytes, erythrocytes, and several types of leucemic cells.

## *Material and Methods*

*Rabbit Cells.*—The rabbit large mononuclear\* and polymorphonuclear phagocytes were obtained from sterile peritoneal exudates. The polymorphonuclear exudates were elicited by intraperitoneal injection of 0.85 per cent NaCl by a method which has been fully described (2). The mononuclear exudates were elicited by intraperitoneal injection of light paraffin oil; this method will be described in a forthcoming publication (3). Each type of cell was washed in sterile 0.85 per cent NaCl and resuspended in the same medium. Small samples of these suspensions were used for the present study, and the remainder were used for phagocytosis experiments. Both types of leucocytes functioned normally in phagocytosis. For the rabbit phagocytes we are indebted to Dr. Balduin Lucké.

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\* No attempt was made at further classification of the large mononuclears by vital staining.

*Human Cells.*—These were obtained by pricking the finger and diluting the resulting drops of blood with Locke solution or 0.85 per cent NaCl solution. These suspensions of unwashed blood cells were used as the aqueous phase in interfacial preparations.

*Mouse Cells.*—For the mouse cells we are indebted to Dr. J. Furth. Mice suffering from lymphoid leucemia (4) were bled from the heart; the blood was diluted with Locke solution, and heparin, (final concentration of heparin, 1:10,000 approximately), was added to prevent clotting. The cells were washed and resuspended in Locke solution. In other instances leucemic lymph nodes were cut up in Locke solution and the cells were washed and resuspended in Locke solution.

*Chicken Cells.*—Chickens, normal, tuberculous, or suffering from myeloid leucemia (5) were bled from the wing vein. The blood was diluted with Locke solution and heparinized; the cells were washed and resuspended in Locke solution. Chicken myeloblasts and myelocytes similar to those used have been illustrated by Ellermann (6). For the chicken cells we are also indebted to Dr. J. Furth.

*Temperature.*—The experiments were conducted at room temperature.

*Interfacial Technique.\**—This is exceedingly simple. A droplet of 0.85 per cent NaCl or of Locke solution containing the cells and a droplet of oil are placed near each other on a carefully cleaned microscope slide. A large cover-slip is laid on top of them; this spreads both drops into films which meet along a line across the slide; the oil tends gradually to displace the water under the cover-glass; the oil-water interface therefore moves slowly in the direction of the aqueous phase. This slowly advancing interface is kept under continual microscopic observation, by the use of a mechanical stage, as it progressively overtakes the cells in the aqueous phase. Information is thus obtained by direct observation regarding the wetting of the cells by both phases at the oil-water boundary; moreover under certain conditions the cells are subjected to compressing or to stretching forces at the interface, so that information regarding their plasticity and fluidity is obtained.

Contamination of the advancing oil-water interface by protein or other adsorbable impurities sometimes causes difficulty. This effect is reduced to a minimum by keeping the test suspension as free as possible of impurities, and by making observations during the first minutes after setting up the preparations.

### *Experimental Results*

*The Observed Behavior of Various Blood Cells at the Oil-Water Interface.*—Observation of various blood cells by the interfacial technique has shown that they fall into two definite categories, *i.e.*, those whose external surfaces are hydrophilic and those whose external surfaces are

\* For description and illustration of this technique, see Mudd, S., and Mudd, E. B. H., *J. Exp. Med.*, 1924, 40, 633, 647; 1926, 43, 127. A modified procedure permitting of more quantitative treatment has recently been developed by Nugent (Nugent, R., *J. Physic. Chem.*, 1931, in press).

relatively hydrophobic. All types of leucocytes studied fall within the hydrophilic group; the outer surfaces of mature mammalian and chicken erythrocytes are relatively hydrophobic. A further subdivision has appeared within each of these groups with respect to the resistance offered to the deforming and stretching forces at the interface.

The cell behavior at the interface will therefore be described under four types, A and B, (hydrophilic cell surface) and C and D, (relatively hydrophobic cell surface). These four types are illustrated in Fig. 1. That cell which most frequently exemplifies the type of reaction under discussion has been used in each instance; every effort has been made to have the drawings correspond as faithfully as possible to the actual appearances under the microscope. It is to be noted, however, that the lines and dots, which are black in the drawing, appear bright against a dark background in the dark-field microscope, with which most of the observations have been made.

It is to be remembered that the slow but sustained movement of the oil-water boundary in the direction of the aqueous phase is an essential feature of the experimental arrangement. In each column of Fig. 1 there is shown the sequence of events as one type of cell is overtaken by this slowly advancing interface.

Type A is most characteristically exhibited by the mononuclear phagocyte, although it is shown also in a smaller percentage of cases by the other types of leucocytes studied. In Fig. 1, A 1, the advancing interface is about to overtake a rabbit mononuclear suspended in the aqueous phase. In A 2 the oil has come into contact with a small part of the surface of the floating cell which it pushes before it. Between A 2 and A 3 the cell is supposed to have touched the microscopic slide, to which it adheres strongly. In A 3 the general line of the advancing interface has passed beyond the cell, but the interface is retarded locally by the adherent cell, from whose surface the oil is unable to displace the water. The cell is thus subjected to a considerable pressure. One effect of this pressure is to tear the cell near its place of attachment to the glass, leaving a little granular cytoplasm behind the interface in the oil phase as shown in A 3 to A 6, inclusive. Another effect of this pressure is to press some of the cytoplasm and its enveloping membrane into rather blunt, stiff projections (A 3 to A 5,

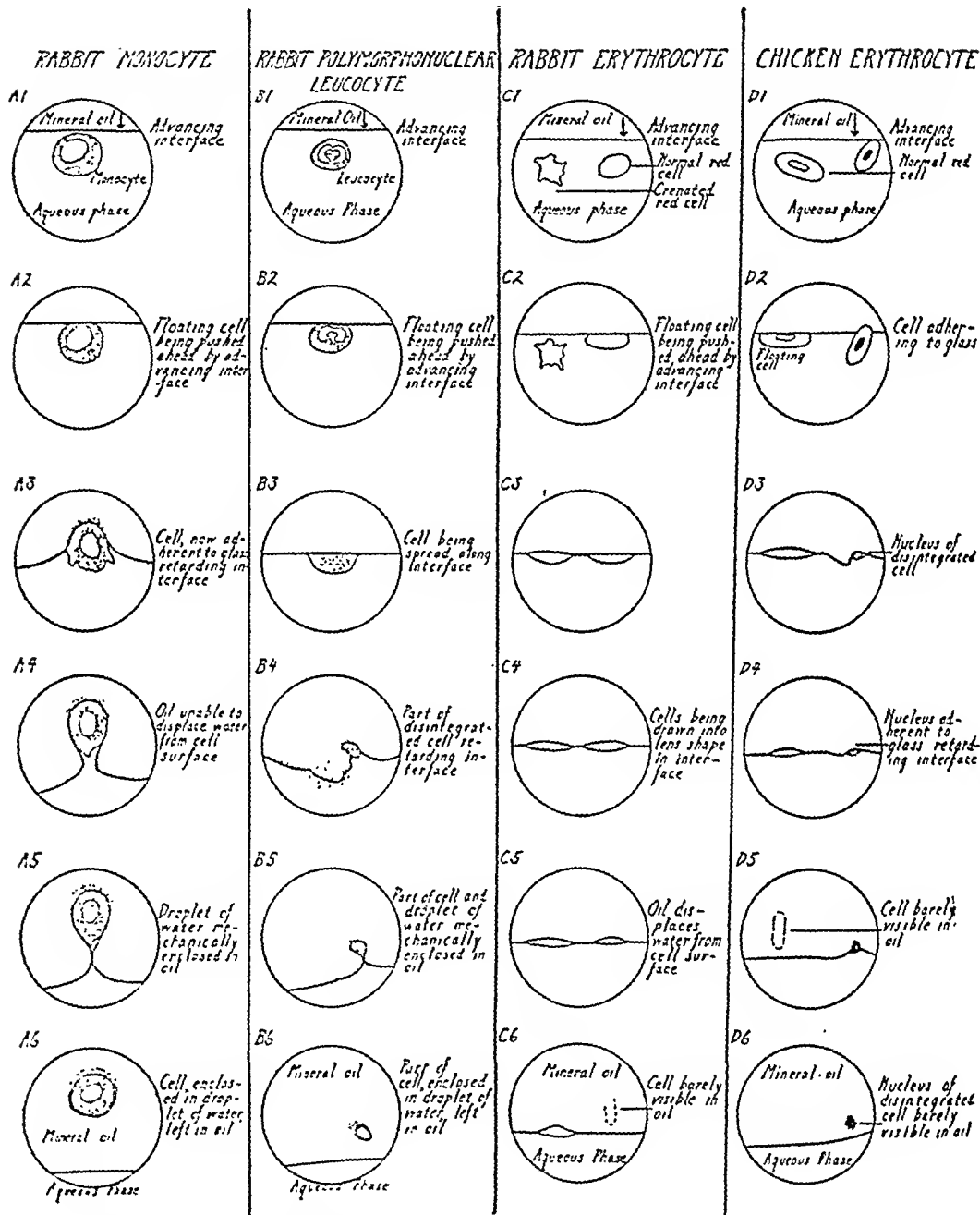


FIG. 1. The behavior of cells at an oil-water interface. Each column represents a typical sequence of events as a cell is overtaken and passed by the advancing interface. For details see text.

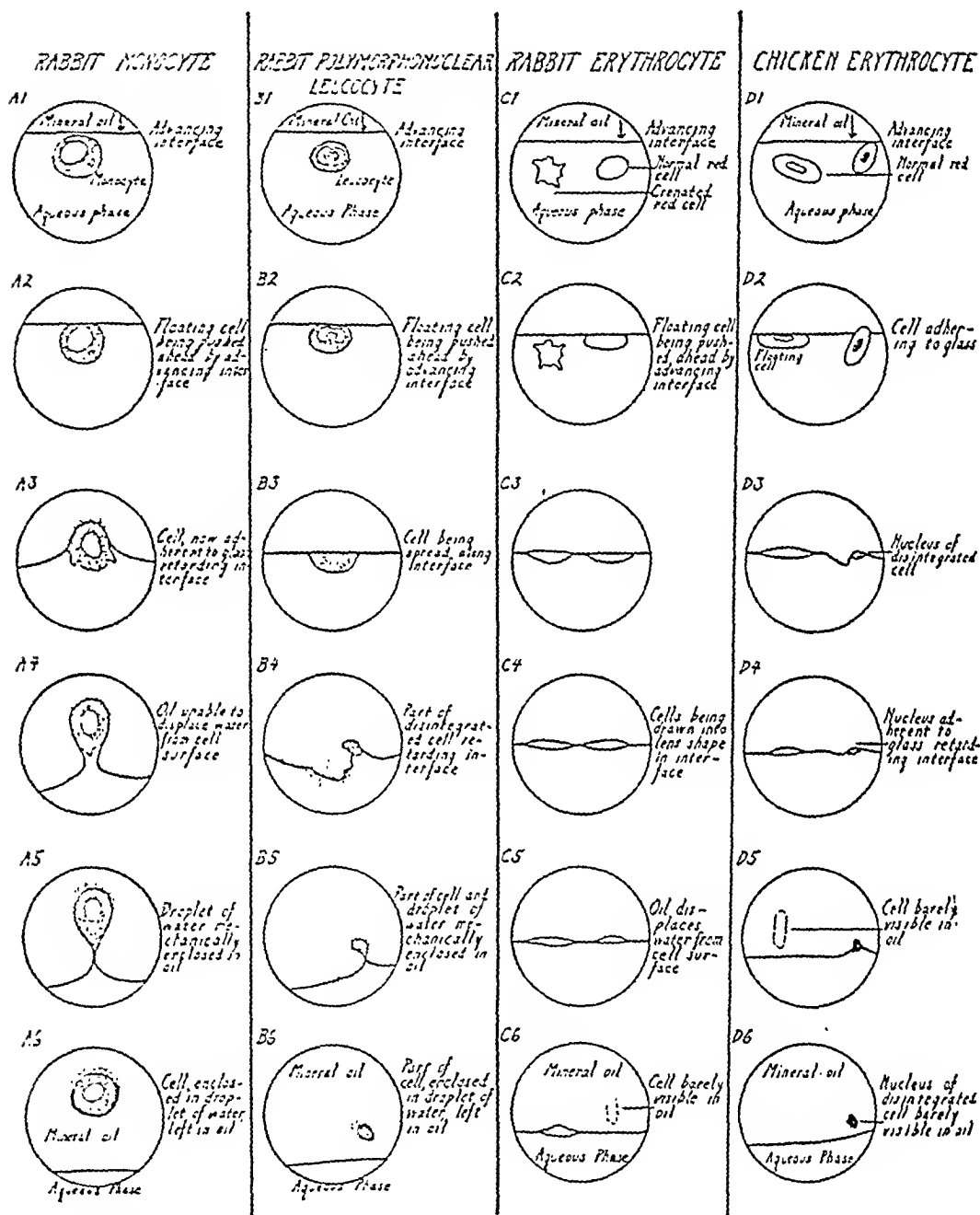


FIG. 1. The behavior of cells at an oil-water interface. Each column represents a typical sequence of events as a cell is overtaken and passed by the advancing interface. For details see text.

inclusive). In A 4 and A 5 one of these pseudopod-like projections is shown, limited by the delicate line of the surface, and filled with an optically clear cytoplasm; the granular cytoplasm has suffered little deformation in spite of the pressure upon it. Such projections, filled with optically clear cytoplasm and enveloped in the surface membrane, are frequently seen when the mononuclears are pressed upon by the advancing interface, often in more striking form than here represented. In A 4 and A 5 the interface has advanced still further, but, failing to displace the water from the cell surface, still suffers local retardation by the adherent cell. The interfaces closing in about the cell from each side are just about to make contact with each other in A 5. Between A 5 and A 6 the contact is supposed to have been made and the new interfacial line quickly returns to a minimal area, (7) leaving behind the cell still enclosed in a delicate envelope of water.

The two most striking features of this sequence are the failure of the oil to displace the water from the cell surface, and the high viscosity of the cell cytoplasm.

In Column B a rabbit polymorphonuclear leucocyte is represented as floating before an advancing interface. In B 2 the oil has made contact with part of the surface and the cell is momentarily carried ahead of the interface. In B 3 the stress along the interface is tending to make the cell lenticular; the nucleus is no longer visible; whether it is broken or is merely obscured by the rapid deformation of the granular cytoplasm we do not know. Unable to withstand the interfacial stress, the cell is torn asunder; the cell detritus lowers certain local interfacial tensions, and the interface runs forward locally (B 4); the visible granules are scattered in the oil and water phases or swiftly swept out of the field along the interface like beads sliding along a string. A portion of the cell usually escapes complete disintegration; such a fragment is shown in B 4 and B 5 retarding the interface. In B 6, as in A 6, a new interfacial line has formed and contracted to minimal area, leaving behind in the oil a fragment of the disintegrated cell still enclosed in a droplet of water.

The features of the typical polymorphonuclear leucocyte (Type B), which contrast with the typical large mononuclear (Type A), are the lower viscosity of the cytoplasm of the polymorphonuclear cell and the lesser resistance of this cell to the stretching forces of the interface.

Both types of leucocyte are alike in the hydrophilic nature of the external surface. Types of behavior intermediate between A and B are of course observed; they are seen with all types of leucocytes, but have been noted as most characteristic of lymphocytes.

Consider now the reaction types with cells of relatively hydrophobic surface properties, the mammalian and chicken erythrocytes. In C 1 a normal and a crenated rabbit erythrocyte are represented as suspended in the aqueous phase. In C 2 the oil has made contact with the normal red cell surface; in C 3 to C 5, inclusive, the red cells are drawn to lens shapes by the interfacial stress; the oil readily displaces the water from the cell surfaces and the cells slip through the interface into the oil without, or with scarcely perceptible, retardation of the interface. A cinematograph of such a sequence with human erythrocytes has been published (8). In the case of the crenated red cell the outer membrane is folded into projections. As this cell enters the interface between C 2 and C 3 the outer membrane is pulled out by the interfacial stress until the folds give way to a smooth contour, (C 3 to C 5, inclusive). This cell also finally slips through into the oil. In the oil the red cells are ordinarily not visible.

The features of this mammalian erythrocyte reaction which deserve emphasis are: first, the ease with which the water is displaced by oil from the cell surface, second, the fluidity of the cell contents; the cell is pulled into a lens by the interfacial stress as a liquid immiscible in both phases would be; and, third, the structure of the outer membrane, which may be folded into projections and stretched to a smooth contour, but which resists disruption by the interfacial stress.

In Column D are represented two chicken red cells, the one normal and floating in the aqueous phase, the other adherent to the glass. In such a preparation the red cells settle out onto the glass within a few minutes, and, as observation continues, more and more of the cells in contact with the glass are hemolysed. Such contact hemolysis by glass has been described for mammalian erythrocytes by Fenn (9). The hemolysed chicken erythrocyte appears in the dark-field to have a pale, silvery outline and a brighter, more granular nucleus than the normal cell. In Fig. 1 the chicken cell in contact with the glass is represented with the granular nucleus, intended to indicate injury, but with the bright golden outline of the unhemolysed cell.



In D 2 the floating cell is momentarily pushed ahead of the advancing interface. Between D 2 and D 3 the floating cell has turned over so that its two larger axes lie in the plane of the boundary surface; hence the cell is viewed in D 3 from its edge. Such an orientation is the usual rule when non-spheroidal objects, such as spermatozoa, spirochetes or bacterial rods, enter the interface. In D 4 this cell is slipping out of the boundary surface into the oil, with scarcely perceptible retardation of the interface.

In D 2 the water is displaced by the oil from one end of the cell adherent to the glass. Between D 2 and D 3 this cell has suddenly disintegrated with consequent local running forward of the interface. The injured nucleus of the disintegrated cell, however, markedly resists entering the oil. This nucleus is represented as having become adherent to the glass between D 3 and D 4. The further advance of the interface is retarded locally in D 5 by the adherent nucleus; however, the interface is represented as pulling over the nucleus in D 6, leaving the nucleus behind in the oil. In other instances the injured nucleus is left behind the interface still enclosed in a droplet of water.

This high resistance to wetting by the oil is believed to be a property of the granular-appearing injured nucleus, in which the protoplasm is probably coagulated. In an uninjured chicken erythrocyte, little or no structure can be seen within the nuclear membrane; when such a cell disintegrates in the interface the nucleus appears to offer comparatively little resistance to passage through the interface.

In most preparations the erythrocytes, both mammalian and avian, are invisible in the oil. Occasionally, however, for reasons that are not wholly clear, the details of the process by which the erythrocyte slips out of the interface into the oil may be made out. The erythrocyte touches and tends to adhere to the glass, the interface passes over that part of the red cell which is in contact with the glass, thus stretching the erythrocyte into a pear-shaped body with the stem attached to the glass under the oil and the base still momentarily in the interface. With further advance of the interface the base of the pear-shaped erythrocyte is pulled out of the interface into the oil and the cell there tends to contract to its usual shape.

The outer surface of the chicken erythrocyte, like that of the mammalian red cell, is relatively hydrophobic. Whether or not the outer

membrane is inherently less resistant than that of the mammalian erythrocyte to disruption by the interfacial stresses we have never been able to determine with certainty. Certainly many of the chicken erythrocytes settled out on the glass do go to pieces in the interface, but the possibility of injury by contact with the glass cannot be excluded. The injured, and probably coagulated nucleus, displays marked resistance to wetting by the oil. The behavior of these injured nuclei in the interface is very much like that of heat-injured

TABLE I  
*Peritoneal Exudate from Rabbit*

| Cell type          | Test oil    | No. of experiments | Reaction types, average | Remarks                     |
|--------------------|-------------|--------------------|-------------------------|-----------------------------|
|                    |             |                    | <i>per cent</i>         |                             |
| Large mononuclears | Triolein    | 3                  | A, 95 B, 5              |                             |
| " "                | Tricaprylin | 4                  | A, 90 B, 10             |                             |
| " "                | Tributyrin  | 2                  | A, 90 B, 10             |                             |
| " "                | Cyclohexane | 3                  | A, 75 B, 25             |                             |
| " "                | Mineral oil | 7                  | A, 85 B, 15             |                             |
| Polymorphonuclears | Triolein    | 3                  | A, 50 B, 50             |                             |
| "                  | Tricaprylin | 6                  | A, 45 B, 55             |                             |
| "                  | Tributyrin  | 2                  | A, 60 B, 40             |                             |
| "                  | Cyclohexane | 5                  | A, 15 B, 85             |                             |
| "                  | Mineral oil | 5                  | A, 15 B, 85             |                             |
| Erythrocytes       | Triolein    | 4                  | C 100                   | Interface slightly retarded |
| "                  | Tricaprylin | 5                  | C 100                   |                             |
| "                  | Tributyrin  | 2                  | C 100                   |                             |
| "                  | Cyclohexane | 3                  | C 100                   |                             |
| "                  | Mineral oil | 2                  | C 100                   |                             |
| Lymphocytes        | Mineral oil | 2                  | A, 80 B, 20             |                             |

human polymorphonuclear leucocytes and heat-coagulated globulins (8, 10).

*The Distribution of the Reaction Types among Various Cells.*—The sources and kinds of cells studied, the oils used as organic phase and the reaction types observed are set forth in Tables I to IV, inclusive. The mammalian erythrocytes all fell within Type C, and with one exception no significant differences were detected with the several oils used. The exception was triolein. The saline-triolein interface

was distinctly, although slightly, retarded in passing over each erythrocyte. This effect could plausibly be attributed to the polar double-bonds of triolein, which doubtless tend to orient themselves toward the water phase; however, we have no further evidence on this point at present.

TABLE II  
*Normal Human Blood Cells*

| Cell type          | Test oil    | No. of experiments | Reaction types, average | Remarks                     |
|--------------------|-------------|--------------------|-------------------------|-----------------------------|
|                    |             |                    | <i>per cent</i>         |                             |
| Polymorphonuclears | Triolein    | 1                  | A, 75 B, 25             | Interface slightly retarded |
| "                  | Tricaprylin | 4                  | A, 20 B 80              |                             |
| "                  | Mineral oil | 2                  | A, 10 B, 90             |                             |
| Erythrocytes       | Triolein    | 1                  | C 100                   |                             |
| "                  | Tricaprylin | 6                  | C 100                   |                             |
| "                  | Mineral oil | 4                  | C 100                   | Some intermediate reactions |
| Lymphocytes        | Tricaprylin | 4                  | A, 40 B, 60             |                             |
| "                  | Mineral oil | 1                  | A, 5 B, 95              |                             |

TABLE III  
*Blood Cells of Lymphoid Leucemic Mouse*

| Source of cells                | Cell type          | Test oil    | No. of experiments | Reaction types, average |
|--------------------------------|--------------------|-------------|--------------------|-------------------------|
|                                |                    |             |                    | <i>per cent</i>         |
| Leucemia blood                 | Polymorphonuclears | Tricaprylin | 3                  | A, 15 B, 85             |
| " "                            | "                  | Mineral oil | 4                  | A, 20 B, 80             |
| " "                            | Erythrocytes       | Tricaprylin | 4                  | C 100                   |
| " "                            | "                  | Mineral oil | 3                  | C 100                   |
| " blood and lymph nodes        | Lymphocytes        | Tricaprylin | 7                  | A, B, and intermediate  |
| Leucemia blood and lymph nodes | "                  | Mineral oil | 8                  | A, B, and intermediate  |

The chicken erythrocytes fell within Type D as already described.

The majority of rabbit mononuclear phagocytes fell within Type A, the minority within Type B; the same was true for the large mononuclear leucocytes of chicken blood. In contrast to this result were

membrane is inherently less resistant than that of the mammalian erythrocyte to disruption by the interfacial stresses we have never been able to determine with certainty. Certainly many of the chicken erythrocytes settled out on the glass do go to pieces in the interface, but the possibility of injury by contact with the glass cannot be excluded. The injured, and probably coagulated nucleus, displays marked resistance to wetting by the oil. The behavior of these injured nuclei in the interface is very much like that of heat-injured

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| "                  | Cyclohexane | 5                  | A, 15 B, 85             |                             |
| "                  | Mineral oil | 5                  | A, 15 B, 85             |                             |
| Erythrocytes       | Triolein    | 4                  | C 100                   | Interface slightly retarded |
| "                  | Tricaprylin | 5                  | C 100                   |                             |
| "                  | Tributyrin  | 2                  | C 100                   |                             |
| "                  | Cyclohexane | 3                  | C 100                   |                             |
| "                  | Mineral oil | 2                  | C 100                   |                             |
| Lymphocytes        | Mineral oil | 2                  | A, 80 B, 20             |                             |

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*Normal Human Blood Cells*

| Cell type          | Test oil    | No. of experiments | Reaction types, average | Remarks                     |
|--------------------|-------------|--------------------|-------------------------|-----------------------------|
|                    |             |                    | per cent                |                             |
| Polymorphonuclears | Triolein    | 1                  | A, 75 B, 25             |                             |
| "                  | Tricaprylin | 4                  | A, 20 B 80              |                             |
| "                  | Mineral oil | 2                  | A, 10 B, 90             |                             |
| Erythrocytes       | Triolein    | 1                  | C 100                   | Interface slightly retarded |
| "                  | Tricaprylin | 6                  | C 100                   |                             |
| "                  | Mineral oil | 4                  | C 100                   |                             |
| Lymphocytes        | Tricaprylin | 4                  | A, 40 B, 60             | Some intermediate reactions |
| "                  | Mineral oil | 1                  | A, 5 B, 95              |                             |

TABLE III  
*Blood Cells of Lymphoid Leucemic Mouse*

| Source of cells                | Cell type          | Test oil    | No. of experiments | Reaction types, average |
|--------------------------------|--------------------|-------------|--------------------|-------------------------|
|                                |                    |             |                    | per cent                |
| Leucemia blood                 | Polymorphonuclears | Tricaprylin | 3                  | A, 15 B, 85             |
| " "                            | "                  | Mineral oil | 4                  | A, 20 B, 80             |
| " "                            | Erythrocytes       | Tricaprylin | 4                  | C 100                   |
| " "                            | "                  | Mineral oil | 3                  | C 100                   |
| " blood and lymph nodes        | Lymphocytes        | Tricaprylin | 7                  | A, B, and intermediate  |
| Leucemia blood and lymph nodes | "                  | Mineral oil | 8                  | A, B, and intermediate  |

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The majority of rabbit mononuclear phagocytes fell within Type A, the minority within Type B; the same was true for the large mononuclear leucocytes of chicken blood. In contrast to this result were

# PROPERTIES OF LEUCOCYTES AND ERYTHROCYTES

TABLE IV  
*Blood Cells of Chicken*

PROPERTIES OF LEUCOCYTES

TABLE IV  
Blood Cells of Chicken

| Condition                         | Cell type          | Test oil    | No. of experiments | Reaction types, average |       | Remarks  |
|-----------------------------------|--------------------|-------------|--------------------|-------------------------|-------|--|
|                                   |                    |             |                    | per cent                |       |  |
| Tuberculous                       | Large mononuclear  | Tricaprylin | 4                  | A, 70                   | B, 30 | In 4 expts. interface retarded   |
|                                   | "                  | Mineral oil | 3                  | A, 70                   | B, 30 |  |
| Normal and tuberculous            | Polymorphonuclears | Tricaprylin | 11                 | A, 40                   | B, 50 | In 3 expts. interface retarded   |
|                                   | "                  | Mineral oil | 7                  | A, 30                   | B, 70 |  |
| Normal, tuberculous, and leucemic | Erythrocytes       | Tricaprylin | 17                 | D 100                   |       | An occasional intermediate reaction  |
|                                   | "                  | Mineral oil | 9                  | D 100                   |       |  |
| Normal and tuberculous            | Lymphocytes        | Tricaprylin | 8                  | A, 80                   | B, 20 | In other experiments differentiation not so clear between myelocytes and myeloblasts |
|                                   | "                  | Mineral oil | 5                  | A, 75                   | B, 25 |  |
| Myeloid leucemia                  | Myelocytes         | Tricaprylin | 2                  | A, 5                    | B, 95 |  |
|                                   | "                  | Mineral oil | 2                  | A, 5                    | B, 95 |  |
|                                   | Myeloblasts        | Tricaprylin | 2                  | A, 95                   | B, 5  |  |
|                                   | "                  | Mineral oil | 2                  | A, 85                   | B, 15 |  |

those with the polymorphonuclear leucocytes of rabbit, man, mouse, and chicken. In each species the behavior of the majority of polymorphonuclear leucocytes corresponded to Type B, that of the minority to Type A. The percentages given in the tables are based on estimates, not on exact counts, and are very rough. However, the accuracy of the estimates is more than sufficient to warrant the conclusion that the polymorphonuclear leucocytes are on the average more fluid cells, less able to withstand the stretching forces of the interface, than the mononuclear leucocytes.\*

Both kinds of rabbit leucocytes disintegrated in the interface in a higher percentage of cases when cyclohexane and mineral oil were used than with the other test oils. This result is in harmony with the fact that the oil-water interfacial tension, and hence the stretching force to which the cells were subjected, (see next section), is extremely high with cyclohexane and mineral oil (12).

The number of observations with normal lymphocytes and with leucemic cells was not so large nor were the differentiations so clear that we care to give them emphasis. It was evident, however, that these cells conformed to the general leucocytic reaction patterns, and had hydrophilic surfaces.

One experiment with the immature leucocytes of a chicken with myeloid leukemia deserves passing mention. In this experiment (Table IV) the very immature cells of the granulocytic series, the myeloblasts, behaved in the interface much like rabbit mononuclears; the mature myelocytes, on the other hand, resembled the polymorphonuclear leucocytes in their interfacial behavior as in their morphology.

*Analysis of Interfacial Tension Relations.*—Since the progressive movement of the oil-water interface brings it successively into contact with the test cells in the aqueous phase, we need not here consider, as in colloidal aggregation problems, the factors influencing probability

\* Whether or not any given cell disintegrated in the interface depended in part upon whether it was freely suspended or adherent to the glass. The adherent cells were more frequently disintegrated than those freely suspended (*cf.* Fenn, 11). Since the mononuclears are larger than the polymorphonuclear leucocytes and readily sedimented out, this effect only serves to make the relatively greater resistance of the mononuclears to disintegration the more striking.

of collision. Let us consider, therefore, the free energy relations when the cell has reached equilibrium in the boundary surface.

When three phases which do not mix are in contact with each other, the three surfaces of separation meet in a line, straight or curved. The case under consideration is that of a cell in the interface between two immiscible liquids. Supposing the cell to be spherical and the surface uniform, the line of contact would form a circle on the surface of this sphere. Let  $O$  (Figs. 2 and 3) be a point in the line of contact, and let the plane of the paper be supposed to be normal to the line at the point  $O$ .

Let  $A$  be the aqueous phase of the preparation; let  $B$  be the organic phase, and  $C$  the disperse phase (cell). We have then three surfaces

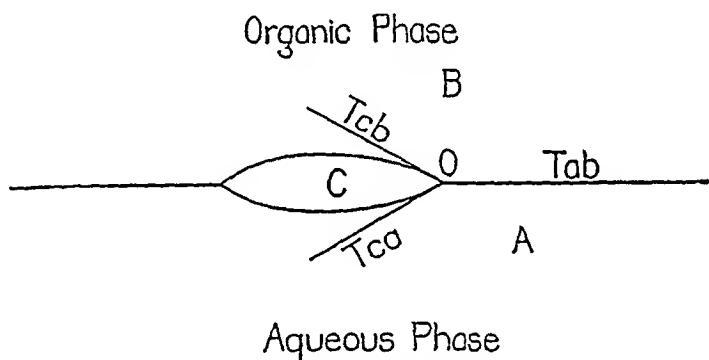


FIG. 2

of separation,  $AB$ , the aqueous-organic phase interface,  $CA$ , the cell-aqueous phase interface, and  $CB$ , the cell-organic phase interface. The corresponding interfacial tensions (*i.e.*, intensity factors of the interfacial free energies) in the surfaces meeting at the point  $O$  are  $Tab$ ,  $Tca$  and  $Tcb$ . Let the angle at which  $AB$  meets the tangent  $CA$  at the point  $O$  be the contact angle,  $\theta$ .

If the disperse phase were a liquid it would in general be deformed by the tension in the surface  $AB$ . If  $Tab > Tca + Tcb$  the liquid droplet would be spread in the interface. If  $Tab < Tca + Tcb$  the droplet would assume some such lens shape as is shown in Fig. 2, the exact angles between the three surfaces of separation being determined by the values of the three interfacial tensions. Such lenses



in air-water interfaces have been measured and figured by Coghill and Anderson (13).

The interfacial technique shows that mammalian red blood cells do actually become lenses in the oil-water interface (see Fig. 1, Column C) and also illustrations from moving pictures of erythrocytes in oil-water interfaces (8). The red cell is obviously not a homogeneous liquid. Its behavior in the interface strongly suggests, however, that it is a liquid surrounded by a surface membrane which is a plastic solid or possibly a liquid of high viscosity.\*

The question naturally arises as to whether the lenticular erythrocyte in the interface represents a true equilibrium of the three interfacial energies. An alternative explanation of its shape would be that the erythrocyte surface was really under a stretching force in the surface  $AB$ , (*i.e.*,  $T_{ab} > T_{ca} + T_{cb}$ ), but that spreading was prevented by the structure of the cell or its surface membrane.  $T_{ca}$  and  $T_{cb}$  are not measurable and offer no help in answering this question. However, the often-repeated observation that the folded surfaces of crenated erythrocytes are pulled out to smooth lenses by the interfacial stress, makes us believe that the surface membrane is really under a stretching force.

The surface of the erythrocyte is evidently not strongly hydrated since it offers only a minimal resistance to passage through the interface into the oil. Nevertheless it does not pass spontaneously out of the interface into the oil. Hence,  $T_{ca} < T_{cb} + T_{ab}$  and  $T_{cb} < T_{ca} + T_{ab}$ .

The interfacial tension relations for the erythrocytes of the chicken seem to be similar to those for mammals.

\* A solid does not undergo permanent change of shape unless the deforming stress exceeds a certain value. A liquid subjected to shearing stress, however small, undergoes continual deformation, although the rate of change of shape may be exceedingly low (14). So far as may be judged by observations of such short duration, the red cell once stretched by the interfacial tension to a smooth lens does not undergo further stretching with time and should therefore be judged to be a solid. The possibility cannot be excluded, however, that longer tension might produce further deformation.

An important paper on Surface films as plastic solids, (Wilson, R. E., and Ries, E. D., in Colloid symposium [monograph, New York, The Chemical Catalog Company, Inc., 1923, 1, 145], should be consulted in this connection.

If the disperse phase is a solid or a jelly too stiff to be readily deformed by the interfacial stresses, it cannot be pulled into a lens. In this case the line of contact of the three surfaces of separation is itself pulled over the surface of the particle until the conditions of equilibrium are satisfied.

At equilibrium  $T_{cb} = T_{ca} + T_{ab} \cos \theta$ .

If  $T_{cb} > T_{ca} + T_{ab}$  the cell will not be in equilibrium in the interface but the line of contact will be displaced toward the surface of greatest tension until the cell is entirely enveloped in the aqueous phase. Similarly were  $T_{ca} > T_{cb} + T_{ab}$  the line of contact would be displaced toward the aqueous side until the particle would be entirely enveloped in the organic phase.

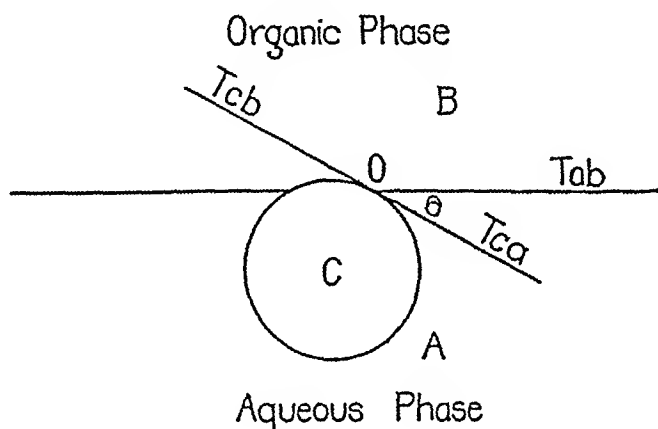


FIG. 3

Thus low tensions in the cell-water interface ( $T_{ca}$ ) tend to bring about a position of the cell in the interface such that the contact angle  $\theta$  is small and the bulk of the cell is in the aqueous phase. Such a position in the interface conversely indicates a low value of  $T_{ca}$  relative to  $T_{cb}$  and strongly suggests that the cell surface is hydrated. It is important to remember, however, that the equilibrium position in the interface indicates the relative values of  $T_{ca}$  and  $T_{cb}$  and not the value of the cell-water interfacial energy alone; it can only be used with this reservation in mind in drawing inferences as to the hydrophobic or hydrophilic nature of the cell surface.

The equilibrium position in the interface of the rabbit large mononuclear leucocyte is approximately that just described. The indication thus given that the surface of the rabbit monocyte has strong

affinity for water is confirmed by the subsequent behavior of the cell toward the advancing interface (See Fig. 1, Column A).

By this method we have not been able to detect or infer differences in the interfacial tensions for the several types of leucocytes. The obvious differences are in the resistance to deformation of the polymorphonuclear and mononuclear cells. The majority of polymorphonuclear leucocytes under the conditions of our experiments were deformed by the interfacial stresses until their internal organization broke down and the cells were disintegrated.

*Attempts to Determine Isoelectric Points of Leucocytes.*—Many efforts were made to determine the isoelectric points of the washed leucocytes in the Northrop-Kunitz cataphoresis cell (15). Cataphoresis of the washed leucocytes suspended in various buffers presented no serious difficulties, and the direction of migration of the leucocytes regularly changed at reactions in the region of pH 4.0 to 4.4. Certain peculiarities, however, aroused suspicion. In the cataphoresis cell containing leucocytes all visible particles and cells moved with practically the same velocity; in the usual cataphoresis experiment, some specks of foreign material are almost always to be seen moving at different velocities, even in the opposite direction, to the principle objects of study. Collodion particles were therefore mixed with the leucocyte suspension and were found to move at the same rate and to reverse direction of migration at the same pH as the leucocytes. Collodion particles alone at pH 4.0 have a high negative charge. It was thus made evident that readily adsorbable substances were present in the suspensions of leucocytes in buffers, probably due to injury of some of the cells. Since we have thus far found no way to avoid this effect we have temporarily abandoned the attempt to determine the isoelectric point of the uninjured leucocyte.

A similar effect with erythrocytes in acid buffers has recently been described by Abramson (16), and has been noted by L. T. Bullock and the writer, studying red cells treated with tannin.

It would seem that such effects have been too little considered in recent experiments directed toward determination of the electrical properties of filterable viruses. When ground-up tissue containing a virus together with a mixture of substances of unknown properties is suspended in a buffer, adsorption complexes of altogether unpredictable

character may be formed. Experiments on the cataphoretic and adsorption behavior of such mixtures should be interpreted with extreme caution.

#### DISCUSSION AND SUMMARY

The resistance to deformation of polymorphonuclear neutrophile leucocytes under the conditions of our observations has been shown to be on the average considerably less than the resistance to deformation of large mononuclear leucocytes. It is recognized of course that the viscosity of leucocytes, as of other cells, may be markedly influenced by osmotic conditions (17), by the reaction of the suspending medium (18, 19), by temperature, or by injury (20, 21). Although the conditions of our observations were quite different from those of the body, they were nevertheless closely similar to those of simultaneous phagocytosis experiments in which the cells functioned exceedingly well (3). Moreover E. R. and E. L. Clark (22) have noted that polymorphonuclear leucocytes in the tails of living tadpoles were more fluid than the macrophages. And Goss (23) in microdissecting human polymorphonuclear neutrophiles reports that they are more fluid than the clasmatoocytes and monocytes studied by Chambers and Borquist (24). Other types of leucocytes have in our experience seemed to fall between the large mononuclear and the polymorphonuclear leucocytes in their average resistance to the interfacial tensions.

The leucocyte of each type studied is surrounded by an exceedingly delicate membrane. This membrane appears under the dark-field microscope as a pale, silvery line not distinguishable by inspection alone from a simple phase boundary between two immiscible liquids. That this is a membrane, however, and not a mere interface between immiscible phases, seems certain. In the first place the cell cytoplasm and the suspending medium are not immiscible. When the cell organization is broken down by the interfacial tension the greater part of the cell contents is immediately dissolved or dispersed. Goss (23) has noted that when the membrane is torn with a microdissection needle disintegration at once spreads over the membrane and the cytoplasm undergoes profound change. Moreover it is improbable that a simple phase boundary could exist in the presence of so much protein, lipid, and other surface active materials as are present in protoplasm; the

tendency of these substances to lower the free interfacial energy must necessarily tend to their adsorption in the interface until, if sufficient material is available at the interface, an adsorption film or membrane may be formed.

Kite (25), in a pioneer microdissection study, described the polymorphonuclear leucocyte as "naked" protoplasm. The contradiction between this statement and those just made is more apparent than real. For the capacity swiftly to form a limiting membrane between itself and other liquids is an attribute of "naked" protoplasm, as has been shown by the beautiful experiments of Chambers (20).

The present study of the wetting properties of leucocytes shows that their external membranes are hydrophilic,\* a character suggesting a surface in which proteins, probably bound water and salts (27), possibly the polar radicles of soaps or fatty acids, rather than non-polar lipid groupings, are predominantly exposed. This makes it the more remarkable that a cell of such fluidity as for instance the polymorphonuclear leucocyte, composed largely of water and of water-soluble materials, should maintain its integrity in an aqueous medium with the aid of a membrane so delicate and so mobile.† The mobility of the membrane, frequently extended in forming new pseudopodia or spreading over the surface of particles being ingested, must require constant entrance into and exit from the membrane of component materials, and their constant reorganization there. The limiting factors in the reformation of such a membrane would be the amounts of adsorbable materials available and their rates of movement up to the surface rather than the time required for orientation there, since the latter phenomenon is exceedingly rapid. Harkins (29), for instance

\* It is well known that the hydration of proteins is related to the pH of the medium. Nugent has shown that this applies also to proteins in thin surface membranes (26). Our experiments with rabbit mononuclear and polymorphonuclear cells were conducted in a faintly acid medium, i.e., 0.85 per cent NaCl in laboratory distilled water; other cells were for the most part studied both in faintly acid (0.85 per cent NaCl) and faintly alkaline (Ringer-Locke solution) suspending media.

† However protoplasm has been likened to liquid crystals by W. T. Bovie and others (28). It is not unlikely that protoplasm owes its integrity to its own internal organization as well as to differentiated surface layers.

has calculated that at a water-water vapor interface at 20°C., from the area occupied by one molecule of water, a molecule would jump out into the vapor and a vapor molecule would fall into this area of the surface 7,000,000 times in one second; the time of orientation of the water molecule he estimates to be of the order of 1/100,000,000 second or less.

The mammalian erythrocyte possesses a surface membrane capable of being folded and of withstanding tension in the interface. This has also been stretched by microdissection needles (21).

The surface of the erythrocyte, as evidenced by its wetting properties, is relatively hydrophobic, relatively non-polar in character, as compared with the leucocyte. Evidence indicating that the erythrocyte surface contains both lipoid and protein components has been summarized in earlier papers (8, 30). We have little to add here other than to point out that the wetting properties of the chicken erythrocyte surface are similar to those fully described for the mammal.

A serious source of error in certain isoelectric point determinations is discussed.

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# SALT EFFECTS ON EGGS AND NAUPLII OF ARTEMIA SALINA L.

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## INTRODUCTION

Among the organisms inhabiting strong brines, the Arthropods are well represented. Crustacea (Abonyi (1), van Doune (2), Schman-kewitsch (11)), Coleoptera (von Lengerken (9), Hase (4)), Diptera (Jones (6)), and Hemiptera (Entz (3)) abound in solutions which often contain more than 25 per cent solids and which should be, according to certain authors (Schmidt (12)), azoic.

While the number of protists with naked cell surfaces is also high in these solutions, and protoplasm should, therefore, be able to absorb water and nutrient from this unusual environment, the arthropods have the advantage that this unusual plasmic mechanism need be confined only to the mesodaeum, as the remainder of the body surface in contact with the *milieu ambiant* is effectively chitinized.

In the case of *Artemia salina*,\* the brine shrimp, used chiefly in our investigations, this can be easily demonstrated by means of vital dyes which, in the uninjured animal, penetrate only the columnar epithelium of the mid-gut (see, for other opinions, Martin and Wilbur (10)). It has been known for a long time that *Artemia* is capable of living for days in solutions of potassium permanganate, potassium bichromate, and silver nitrate.

This fact might suggest that the organism is particularly resistant to these substances; a conclusion, however, that is not warranted by the facts. For, as Dr. Herbert Warren has shown in this laboratory, the organism possesses in the muscular mechanism of its loop-shaped proctodaeum a most efficient means of hermetic closure of the gut.

\* For the extensive literature, see Abonyi (1).

Experimentally, this may be tested by placing the animals in sea water to which a fine suspension of barium carbonate has been added. The presence of this substance in the gut can be immediately ascertained, and it was found that actual ingestion of the particles was often delayed for more than 2 days after the animals were placed in the solution.

*Artemia*, therefore, has a means of checking effectively the passive intake of the liquid environment (it is a *Strudler*, in the sense of Lang).

If we want to test the effect of various substances in the environment upon *Artemia*, the voluntary closure of the proctodaeum might obscure the results to a marked degree. The variable results obtained by us with adult shrimps may be chiefly ascribed to this factor.

As the gut is formed in the late nauplius stage, before the second instar, it seemed advisable to work with the nauplius stage exclusively.

The eggs of *Artemia* are available, the year around, in huge quantities. In the spring when the salt crust in the salines becomes dissolved and the environment reaches a favorable salinity, the "winter eggs" of the shrimp swell and burst. The nauplius, after having passed through about fourteen instars (Heath (5)), reaches maturity and produces eggs. By this time, the pool has become more concentrated with the advanced season. The eggs float on the dense brine, the wind drifts them leeward, and by further evaporation of the pool the eggs are beached in long windrows, often about a half inch thick and 3 to 4 feet wide.

The yield in eggs of a two-acre saline near Marina, California, over the 1928-29 season, was conservatively estimated by us at 100 pounds. About twenty pounds of eggs were cleaned by screening and washing, and showed, even after one year, almost 100 per cent "germination." *Artemia* is, therefore, available as a homogeneous and easily transported material and is well worthy, because of its curious behavior, to become a physiological standard object.

### *Ecdysis*

The eggs are spheroidal, somewhat angular objects; in a dry condition, often with a concavity. They average  $200\mu$  in diameter. The color varies from creamy white to almost blackish brown. A mass of the eggs has a sepia brown color. Spangenberg (13), who described the ecdysis of *Branchipus* (*Branchinecta*), points out that the process in *Artemia* is quite similar and that the eggs possess two membranes. The outer (*Eikapsel*) is hard and unyielding, chiefly composed of chitin; the inner (which arises from the chorion) is very thin and yielding, and is proteinaceous in nature. When *Artemia* eggs are placed in sea water, the outer capsule will burst after about 20 hours at room temperature. The nauplius will be still enclosed in the inner membrane, which is still attached to the outer capsule by the chorion, but hangs down from it, as Spangenberg says "wie die Gondel am

Ballon." After a few hours, the thin membrane (called "membrane" in this paper) ruptures and the nauplius swims around.

It was found, early in this investigation, that the ecdysis of the inner membrane could be incited by a much wider variety and range of solutions than the appearance of living nauplii. The second process, while dependent upon the first, can only be caused by solutions of very definite composition, showing that *Artemia*, instead of being indifferent to chemical environment, is indeed very sensitive to it.

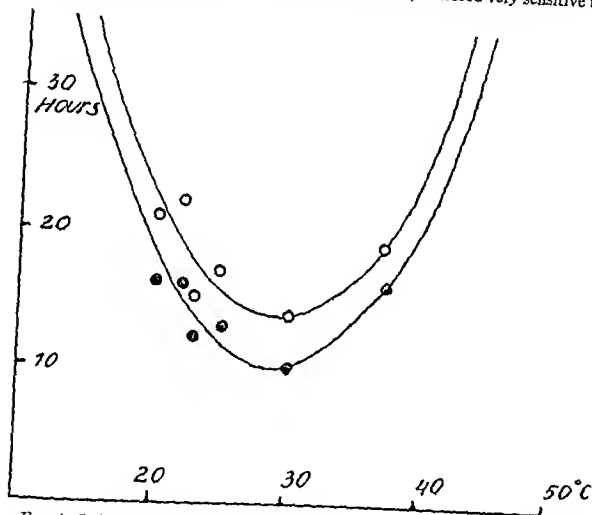


FIG. 1. Influence of temperature on the incubation time of nauplii (solid dots) and on membrane formation (open circles).

The rapidity of both membrane formation and the ecdysis of the nauplius is markedly influenced by temperature. Fig. 1 shows that optimal temperature for both processes lies around 30°, although the speed is still considerable at 22°C. Eggs were placed in 50 cc. flasks in the solution to be tested, and incubated at 22°C. The frequency and speed of both membrane formation and nauplii ecdysis were noted several times during the day. As abnormalities, we observed in certain solutions the loosening of the inner membrane prior to the appearance of the nauplius and the mass death of nauplii immediately after ecdysis.

*The Influence of Hydrogen Ion Concentration*

As it was found that the eggs hatched well in pure NaCl solutions and also in phosphate, mixtures were made of NaCl 1 mol,  $\text{NaH}_2\text{PO}_4$  1/30 mol,  $\text{Na}_2\text{HPO}_4$  1/30 mol, HCl 0.1 mol, and NaOH 0.1 mol. Membranes were formed in the entire range of pH 1.7 to pH about 13 (0.1 mol NaOH). Nauplii hatched in 0.1 mol NaOH and also in very acid solutions (pH = 2). It is hardly probable, therefore, to support the contention of Labbé (7), who claims that the distribution of the

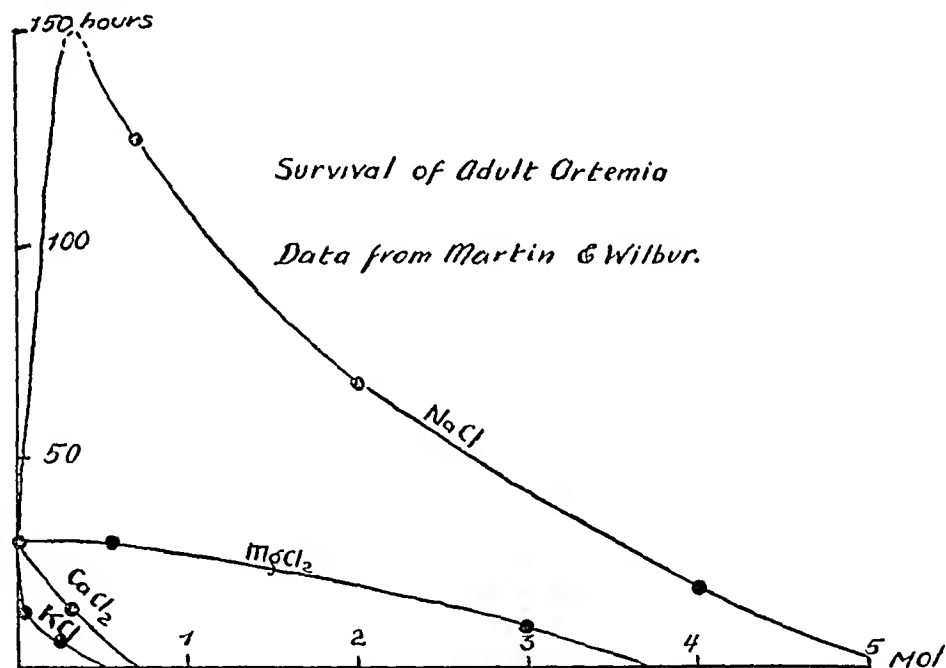


FIG. 2. Survival of adult *Artemia* as a function of salt concentration (after Martin and Wilbur).

brine organisms is dependent upon pH. Moreover, evidence is accumulating to show that the limiting influence of acids and bases depends more upon the concentration of the undissociated acids than upon the pH.

*Effects of Concentration*

As Martin and Wilbur (10) observed, the adults of *Artemia* survive in solutions of  $\text{MgCl}_2$ ,  $\text{CaCl}_2$ , HCl, and NaCl. The astonishing fact

TABLE I

|                                       | Membrane<br>formation | Swimming<br>nauplii | Toxic to mem-<br>brane<br>forma-<br>tion | Toxic to<br>nauplii | Toxic to both<br>mem-<br>brane<br>formation<br>and<br>nauplii | Mem-<br>brane<br>sepa-<br>rated |
|---------------------------------------|-----------------------|---------------------|--|---------------------|---|---------------------------------|
| Sea water.....                        | +                     | +                   | —  | —                   | —   | —                               |
| Urea.....                             | 0 01-1 5 M*           | 0 01-0 6 M          | —  | 0 7 M               | —   | 0 01                            |
| NaCl.....                             | 0 01-3.5              | 0 01-3.5            | 2 5 M                                    | —                   | 4.0   | 0.01                            |
| NaCNS.....                            | 0 01-0 78*            | —                   | —  | 0 01                | —   | —                               |
| NaBr.....                             | 0 01-2 0*             | 0 01-2 0*           | —  | —                   | —   | 0.01                            |
| NaI.....                              | 0 01-0 7*             | —                   | 0 01                                     | 0 01                | —   | —                               |
| Na <sub>2</sub> SO <sub>4</sub> ..... | 0 01-2 0              | 0 01-2 0*           | —  | —                   | —   | 0 01                            |
| NaNO <sub>3</sub> .....               | 0 01-0.70*            | 0.01-0 70*          | —  | 0 56                | —   | —                               |
| KCl.....                              | 0 01-0.78*            | —                   | —  | 0 01                | —   | 0.01                            |
| KI.....                               | 0.01-0 70*            | —                   | 0 01                                     | —                   | 0 01  | 0 01                            |
| KBr.....                              | 0.01-0 70*            | —                   | —  | 0 01                | —   | 0 01                            |
| K <sub>2</sub> SO <sub>4</sub> .....  | 0.01-0 50             | —                   | —  | 0.10                | 0.50  | 0.10                            |
| KNO <sub>3</sub> .....                | 0 01-0.70*            | —                   | —  | 0 01                | 0 65  | 0.10                            |
| LiCl.....                             | 0 01-0.78*            | —                   | —  | 0 01                | —   | 0.01                            |
| RbCl.....                             | 0.28*                 | —                   | —  | —                   | 0 28*   | 0.28*                           |
| NH <sub>4</sub> Cl.....               | 0 01-0 78*            | —                   | —  | 0.01                | —   | 0 01                            |
| BaCl <sub>2</sub> .....               | 0 01-1 4*             | —                   | —  | 0 01                | —   | 0.15                            |
| CaCl <sub>2</sub> .....               | 0 01-1.7              | 0 01-1 4            | 1 8                                      | 1 5                 | 1 8   | 0.01                            |
| MgCl <sub>2</sub> .....               | 0 01-1.5              | 0.01-0 8            | 1 6                                      | 0.9                 | 1 6   | 0.01                            |
| SrCl <sub>2</sub> .....               | 0 01-1 2              | 0 3-0 6             | 1 3                                      | 0 65                | 1.5   | 0 55                            |

\* Highest concentration tried.

TABLE II

| Salt                                  | Membrane | Nauplii | Remarks          |
|---------------------------------------|----------|---------|------------------|
| NaCl.....                             | 0 5      | 0.35    | Survival >6 days |
| NaBr.....                             | 0.45     | 0 1     | Survival >6 days |
| NaNO <sub>3</sub> .....               | 0.5      | 0.1     | Survival >6 days |
| Na <sub>2</sub> SO <sub>4</sub> ..... | 0.42     | 0.2     | Survival >6 days |
| NaI.....                              | >0.7     | ....    |                  |
| NaCNS.....                            | 0 6-0.7  | ..      |                  |
| KCl.....                              | 0 38     | ..      |                  |
| KBr.....                              | >0 7     | ....    |                  |
| KI.....                               | 0.4      | ..      |                  |
| NH <sub>4</sub> Cl.....               | 0 40     | ..      |                  |
| CaCl <sub>2</sub> .....               | 0 20     | 0 40    | Survival 4 days  |
| MgCl <sub>2</sub> .....               | 0.36     | 0.45    | Survival 4 days  |
| SrCl <sub>2</sub> .....               | 0 20     | 0 50    | Survival 2 days  |
| BaCl <sub>2</sub> .....               | 0 1-0.2  | ....    |                  |
| Urea.....                             | 1.20     | 0.40    | Survival 2 days  |

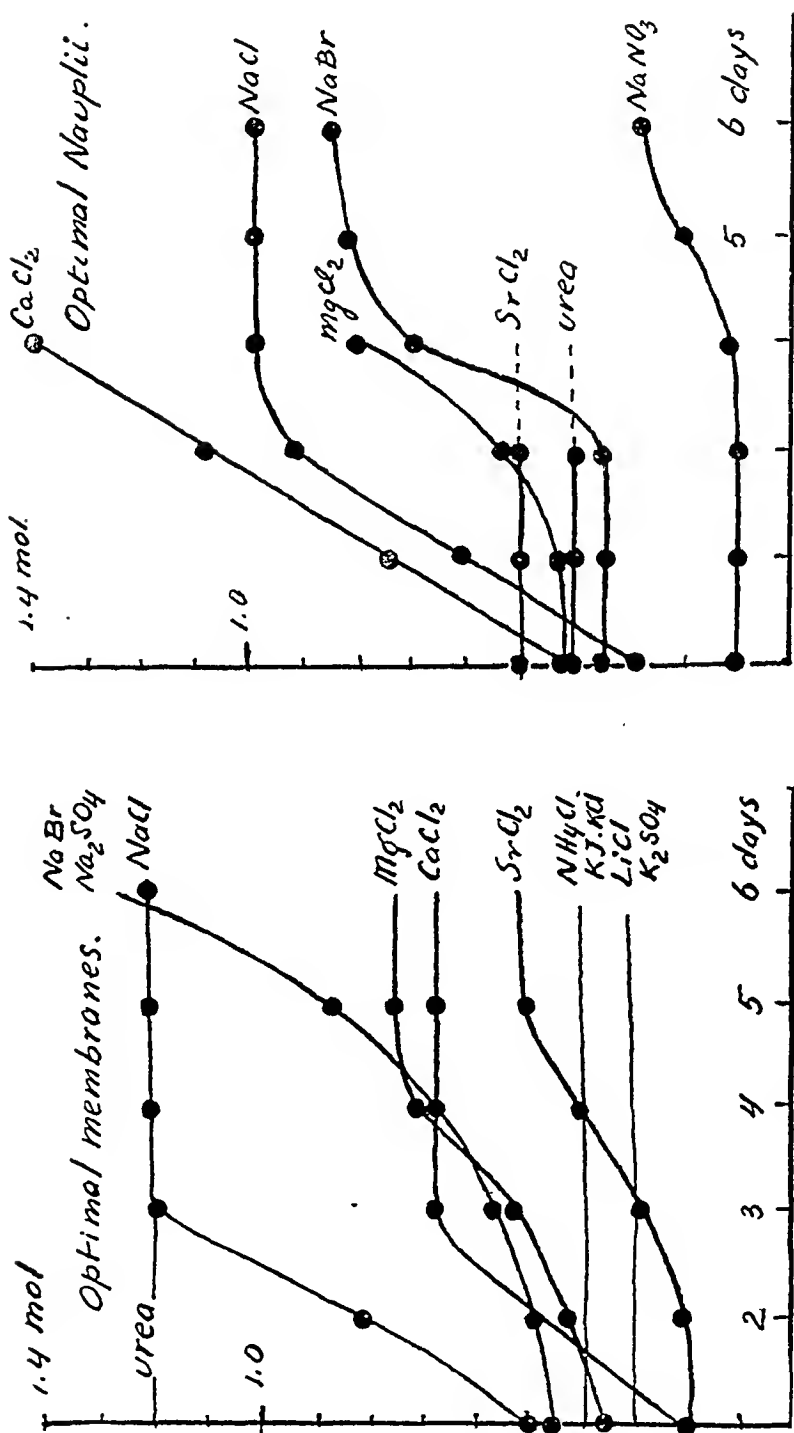


FIG. 3

Fig. 3, Optimal membrane formation in various solutions as a function of time

FIG. 4

Fig. 4, Optimal development of nauplii in various solutions as a function of time

was brought out that KCl was very toxic (see Fig. 2). This fact was substantiated by us, for the nauplii failed to develop in solutions containing more than 0.1 mol KCl. The following table gives the results of our work with solutions of simple salts. In certain solutions, where bacterial growth was apt to occur within 2 days (Urea,  $\text{NaNO}_3$ ), the results may be influenced by this factor.

The incubation time is, in some instances, dependent upon concentration; but the osmotic effect (if present) is entirely overshadowed by the chemical influence of the particular salts. Figs. 3 and 4 show the maximal appearance of membranes, in respect to nauplii in various concentrations of the same salt, as a function of incubation time. Table II shows the "optimal" concentrations (highest frequency of membranes and nauplii formed) for various salts.

When we take from this table only those solutions that support life, we find:

| Average for          | Optimal<br>for membrane<br>formation | Optimal<br>for Nauplii |
|----------------------|--------------------------------------|------------------------|
|                      | mol                                  | mol                    |
| Na salts.....        | 0.48                                 | 0.24                   |
| Alkaline earths..... | 0.23                                 | 0.45                   |
| Urea.....            | 1.20                                 | 0.40                   |

With the exception of  $\text{Na}_2\text{SO}_4$ , which showed high values (0.42 mol, 0.2 mol) membrane formation might be caused, at least partly, by osmotic processes, as the values increase with diminished dissociation. No such osmotic factor is suggested for the appearance of nauplii, however. The range of NaCl concentrations suitable for life in *Artemia* (0–3.5 mol) is so great, moreover, that it would be difficult to account for this phenomenon by osmotic theory.

As remarked before, *Artemia*, far from being indifferent to the chemical environment, is intensely specialized. Sodium salts alone appear to be the most favorable, and the anions also play an important rôle. There actually remain only NaCl and NaBr as solutions capable of causing normal ecdysis; for even the chlorides of Ca and Mg, while able to permit life for a few days, are definitely toxic.

### Antagonisms

Early in our work, it was found that filtered sea water, to which traces of  $\text{KNO}_3$  and  $\text{K}_2\text{HPO}_4$  were added (in order to induce the development of algae) was a convenient solution for bringing *Artemia* to maturity *ab ovo*. Sea water contains K, Mg, and Ca and, moreover, potassium salts were added.

The natural environment of *Artemia* is often very rich in magnesium, so the fact suggested itself that a certain antagonism should be present.

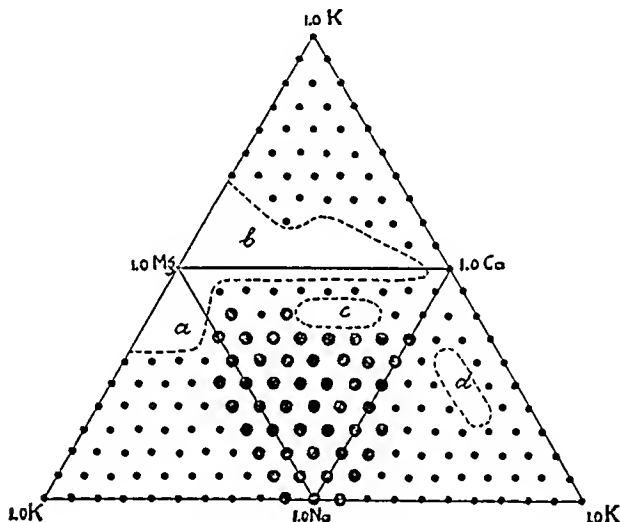


FIG. 5: Diagram illustrating salt antagonism in *Artemia*. The small dots represent membrane formation, the large dots represent nauplii. In the areas *a*, *b*, *c*, and *d* no development took place.

In our work, we have tried only three and four salt combinations, namely, the chlorides of Na, K, Mg, and Ca, always to a total molarity of 1 M.

The objection may be raised that this gives only a one-sided picture, inasmuch as the nature of the antagonism may vary with the concentration (as one of us has demonstrated for *Dunaliella*). Also, it gives no picture of the influence of anions, which is apparently secondary but not negligible. However, we had to stay within the normal limits of experimental possibilities, as the present study already involved several thousand cultures.

Fig. 5 gives the result of the work with three salt combinations.



The small dots represent salt combinations in which membrane formation alone was observed; the heavy dots represent combinations in which nauplii appeared and remained alive for several days.

It may be seen directly that sodium is able to antagonize K in proportion of 0.9 to 0.1 molar. This antagonism persists after addition of Mg up to 0.5 mol  $\text{MgCl}_2$  and, upon addition of  $\text{CaCl}_2$  up to 0.3 molar. In the areas *a*, *b*, *c*, and *d*, no development of any kind took place. In order to obtain the complete four-salt picture, the three outer triangles in Fig. 5 may be rotated on their central sides, making the three points (marked K) coincide. In that way, a tetrahedron is

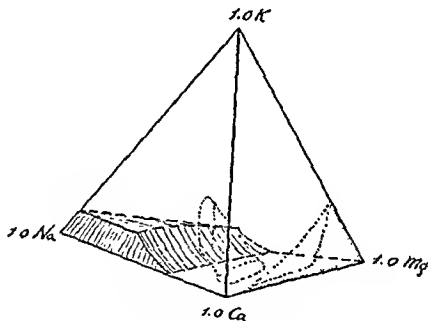


FIG. 6. Three dimensional diagram showing range of development of *Artemia* nauplii.

formed and it remains only to determine the physiological possibilities of the combinations represented by points inside that solid. Instead of tabulating the results obtained, we shall represent them diagrammatically in Fig. 6. Here, the dotted lines delimit the "forbidden areas," while the shaded area represents the salt combinations in which nauplii appear and remain alive for several days. The remainder of the tetrahedron represents salt combinations in which membrane formation, only, was observed.

*Artemia* represents, therefore, an organism that is very sensitive to potassium salts. The toxic effect of these salts may be antagonized to

a small degree by sodium and, to a lesser extent, by magnesium and calcium. Martin and Wilbur (10), who did the pioneer work on salt antagonism in *Artemia*, report that "in two experiments, one with 0.1 mol NaCl + 0.2 mol MgCl<sub>2</sub>; the other with 1.38 mol NaCl + 0.162 mol MgCl<sub>2</sub> + 0.03 mol CaCl<sub>2</sub>, mobility persisted more than 300 hours on the average. No other mixtures gave better results than pure NaCl solutions."

The solutions described by these authors are all situated close to the point in Fig. 4 marked 1.0 Na. It is, therefore, possible that the adult has slightly more restricted salt requirements than the nauplius. It must be pointed out, however, that survival time of adults is not a good quantitative measure, as explained earlier in this paper.

### *Artemia and Its Natural Environment*

During the evaporation of sea water in the solar salines, *Artemia* appears at an early stage. The reason why it does not occur in the open ocean is probably a question of food, combined with its total defenselessness.\* *Artemia* continues to increase until most of the CaSO<sub>4</sub> is precipitated from the sea water. (When the "brine worms" die, the brine is transferred from the pickle pond to the saltern.) At this point, the molarity in NaCl is about 2.7 and, therefore, not high enough to cause death. The potassium increases up to 0.1 molar only in very concentrated brines, so this factor may hardly account for the death of the shrimp. Inasmuch as *Artemia* disappears together with *Dunaliella*, its principal food, this might account for its disappearance, although the red bacteria, which cause the red coloration of the bitterns, may be used to a certain extent. It is most probable, however, that the increasing Mg content of the brine is more important. This factor, combined with the sudden depletion of the food supply, might well account for the disappearance of the "brine worm."

The distribution of *Artemia* in the inland salines checks well with the facts observed in the laboratory. In desert lakes, rich in potassium, *Artemia* is signally absent; while *Ephydra*, the brine fly, occurs in great masses. In Great Salt Lake, which is chemically like a sea

\* Students of Professor G. E. MacGinitie report *Artemia salina* in a haul from Elkhorn Slough, California, which is directly connected with the open ocean.

water concentrate, *Artemia* is present, also in the small "playa"-like pools near Marina, California, which contain a solution of NaCl and NaHCO<sub>3</sub> with very little potassium.

#### SUMMARY

Eggs of *Artemia salina* L., the brine shrimp, are easily obtainable in large quantities. Ecdysis takes place in two stages: (a) extrusion of the inner membrane, and (b) ecdysis of the nauplius from that membrane. The conditions which allow for the former are much more varied than those for the latter.

Nauplii form in only solutions of a few sodium salts; and, in Mg, Ca, and Sr salts, potassium is very toxic.

The possible environment for the nauplii (1 M total molarity) has been ascertained for chlorides of Na, K, Mg, and Ca.

The facts observed account for the peculiar distribution of the organism.

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# SALT EFFECTS ON SWARMERS OF *DUNALIELLA VIRIDIS* TEOD.

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The green polyblepharid flagellate *Dunaliella viridis* occurs in highly concentrated salt solutions the world over (for a description and further literature see (2)).

It is a naked, green biflagellate ( $12 \times 8\mu$ ), with a cupshaped chromatophore and a red eye-spot. The nucleus is situated in the colorless anterior portion. In the posterior part we find a doughnut-shaped starch-body and a pyrenoid, which is often situated in the centre of the starch-body. In a previous communication (2) it could be established that this flagellate must be looked upon as an organism the outer surface of which has lyophilic properties and a pronounced negative charge.

This charge may be related to the toxic effect exerted by calcium and magnesium salts which, in rather low concentrations, cause the disorganisation of the organism. The environment of the organism, however, often contains considerable quantities of both these metals. We know, to be sure, of a great many alkaline lakes in which only traces of Ca and Mg are present; but in the evaporation of sea water, in the range of densities in which *Dunaliella* thrives, these elements form a considerable percentage of the total dissolved matter, as Table I (recalculated from Usiglio (7)) shows.

Even in strong bitterns, chiefly containing magnesium sulfate and chloride, the flagellate appeared after several months of incubation.

In order to test the effect of various quantities and proportions of Na, Mg, and Ca, two strains of *Dunaliella* (one from Marina, California, marked *M* and one from Sand Springs, Nevada, marked *S*) were transferred to two parallel series, one at a pH of 6; the other kept at a pH of 9 by means of K-phosphate mixture. All cultures contained,

moreover, 0.02 per cent  $\text{Na}_2\text{SO}_4$  and 0.02 per cent  $\text{KNO}_3$ . Table II shows the combinations of the chlorides used in the experiment.

TABLE I

| Cations in sea water—evaporates (after Usiglio) |                |                |                |   |
|---|----------------|----------------|----------------|---|
| Specific gravity                                | Molarity in Na | Molarity in Mg | Molarity in Ca | $\frac{[\text{Mg}^{++}]}{[\text{Ca}^{++}]}$ |
| 1.026   | 0.50           | 0.055          | 0.011          | 5.0   |
| 1.050   | 0.95           | 0.105          | 0.019          | 5.5   |
| 1.126   | 2.63           | 0.289          | 0.035          | 8.3   |
| 1.214   | 3.80           | 0.426          | 0.018          | 23.7  |

TABLE II

*Molarity of Cations Used in Antagonism*

| Na | Mg  | Ca | Na | Mg | Ca   | Na | Mg  | Ca   | Na   | Mg   | Ca   |
|----|-----|----|----|----|------|----|-----|------|------|------|------|
| 1  | 0   | 0  | 1  | 0  | 0.01 | 1  | 0.1 | 0.01 | 1    | 0.2  | 0.1  |
| 2  | 0   | 0  | 2  | 0  | 0.01 | 2  | 0.1 | 0.01 | 2    | 0.2  | 0.1  |
| 3  | 0   | 0  | 3  | 0  | 0.01 | 3  | 0.1 | 0.01 | 3    | 0.2  | 0.1  |
| 4  | 0   | 0  | 4  | 0  | 0.01 | 4  | 0.1 | 0.01 | 4    | 0.2  | 0.1  |
| 1  | 0.1 | 0  | 1  | 0  | 0.1  | 1  | 0.2 | 0.01 | 1.67 | 0    | 0.67 |
| 2  | 0.1 | 0  | 2  | 0  | 0.1  | 2  | 0.2 | 0.01 | 1.67 | 0.7  | 0.60 |
| 3  | 0.1 | 0  | 3  | 0  | 0.1  | 3  | 0.2 | 0.01 | 1.67 | 0.14 | 0.53 |
| 4  | 0.1 | 0  | 4  | 0  | 0.1  | 4  | 0.2 | 0.01 | 1.67 | 0.20 | 0.47 |
| 1  | 0.2 | 0  | 1  | 0  | 0.2  | 1  | 0.5 | 0.01 | 1.67 | 0.27 | 0.40 |
| 2  | 0.2 | 0  | 2  | 0  | 0.2  | 2  | 0.5 | 0.01 | 1.67 | 0.34 | 0.33 |
| 3  | 0.2 | 0  | 3  | 0  | 0.2  | 1  | 0.1 | 0.1  | 1.67 | 0.41 | 0.27 |
| 4  | 0.2 | 0  | 4  | 0  | 0.2  | 2  | 0.1 | 0.1  | 1.67 | 0.47 | 0.20 |
| 1  | 0.5 | 0  | 1  | 0  | 0.5  | 3  | 0.1 | 0.1  | 1.67 | 0.54 | 0.13 |
| 2  | 0.5 | 0  | 2  | 0  | 0.5  | 4  | 0.1 | 0.1  | 1.67 | 0.60 | 0.07 |
|    |     |    |    |    |      |    |     |      | 1.67 | 0.67 | 0.0  |

A total of  $2 \times 2 \times 57 = 228$  cultures

The algae were grown, aerobically, in 50 cc. flasks, in the "light box," under continuous illumination\* at a temperature varying between 22°C. and 24°C.

\* The box is 50 cm. square, 80 cm. high. Central illumination is supplied by three 40 watt lamps.

The series was started January 18, 1930 and examined at regular intervals until July, 1930.

In the Sand Springs salt (collected November, 1926), apparently only *Dunaliella* cysts and a few fungus spores were left; for no other organisms appeared in the cultures. From the salt from a small lagoonal pond near Marina, California, collected July, 1929, several organisms could be cultured besides the *Dunaliella viridis*. There appeared in certain cultures:

- (a) Orange cysts, reminiscent of *Dunaliella salina*.
- (b) Pink bacteria (Peirce 6).
- (c) Fungi.
- (d) A blue-green alga (*Gloeothecae* ? or *Rhabdoderma* ?).
- (e) A colorless flagellate (*Amphimonas* ?).

The cultures from Marina gave, therefore, a better picture of the ecological assembly in the natural environment than the cultures from Nevada.

Within two weeks, the following cultures from Marina and Sand Springs showed copious development, independent of concentration of sodium chloride:

|                                 |                          |
|---------------------------------|--------------------------|
| pH 9, 1, 2, 3, and 4 molar NaCl | no magnesium, no calcium |
| " " " " " " " " " "             | 0.01 mol Ca              |
| " " " " " " " " " "             | 0.1 mol Mg no calcium    |
| " " " " " " " " " "             | 0.1 mol Mg 0.01 mol Ca   |

There was, however, considerable variation, even within this series of four. In no instance did the incubation time markedly vary with the NaCl concentration. In any molarity of NaCl, very rapid development took place (marked increase within 3 to 6 days after inoculation). While both the magnesium and calcium retarded the development markedly, the calcium, was the more toxic of the two, as could be predicted from previous experience. The cultures containing both magnesium and calcium developed in a very short time. So it was thought that, perhaps, the two metals might be antagonistic in their effect upon *Dunaliella*. It must be remembered, however, that in an organism like *Dunaliella*, both morphologically and physiologically plastic, the term "toxic" applies only with certain reservations. For the powers of adaptation of this flagellate are great and it is not unlikely that even in solutions high in calcium and magnesium, it might develop, be it after a very long period of time.

The antagonisms, as described originally by Loeb (4) and others, are, in a way, more "dramatic" in their action. So also are the cal-

cium-magnesium antagonisms, as described by Meltzer and Auer (5) and, more recently, by Gellhorn (3). To be sure, the all-or-nothing principle applied more or less to the experimental series described here, inasmuch as certain combinations of salts seemed to be utterly unsuitable; still, we have to remember that our experiment lasted only six months.

While taking incubation time as a measure of toxicity of an environment, we are aware of the inadequacy of this yardstick. For the retardations in development may be due to "previous experience" of the organism which, both as a colloid and as a living being, reflects its earlier history into the "new" environment. The strain from Marina had passed only one transfer with heavy infection on an artificial medium while the Sand Springs strain had passed two transfers on the same medium, which had the following composition\*:

| <i>Tapwater</i>                       |                 |
|---------------------------------------|-----------------|
|                                       | <i>per cent</i> |
| KNO <sub>3</sub> .....                | 0.05            |
| K <sub>2</sub> HPO <sub>4</sub> ..... | 0.05            |
| MgSO <sub>4</sub> .....               | 0.05            |
| NaHCO <sub>3</sub> .....              | 0.1             |

Salts to a final concentration of 15 per cent.

pH adjusted to about 9.

The behavior of both strains showed a rather striking parallelism, which encouraged me to believe that the results express a rather general response of *Dunaliella* towards its environment.

*The Measurement of Antagonism.*—It would be futile to measure antagonism by a statistical treatment of the results obtained, because of the great number of variables introduced into the experiment. The variable factors are: acidity, incubation time, molarity in sodium, in magnesium, and in calcium.

It was thought advisable to group three variables together, namely, incubation time, magnesium, and calcium concentration. The way in which the degree of antagonism was determined is shown in Fig. 1.

The plotting of the effects of antagonism may be done by means of a phase-diagram if the variables are homogeneous. However, if we

\* Suggested by Dr. C. B. van Niel.



have three heterogeneous variables as in the present case, another method may be used to advantage. Fig. 1 represents, on the ordinate, the effect on incubation time of concentrations of a substance  $a$ ; on the abscissae, the effect of concentrations of a substance  $b$ . A biological variable, for instance incubation time, is introduced.

If a concentration of  $b$  alone causes an incubation time of  $b_2$  and a concentration of  $a$  alone causes an incubation time of  $a_1$  and the two

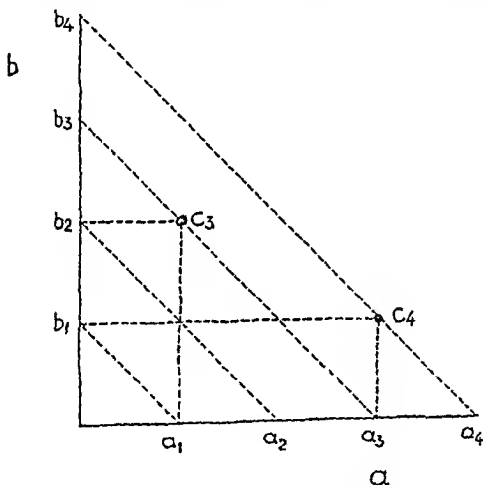


FIG. 1. Influence of salts  $a$  and  $b$  upon incubation time (dotted lines). Effect additive; no antagonism.

substances show no antagonistic action we may expect their action to be additive. The point  $c_3$  represents, therefore, the combined effect of the two salts. This point  $c_3$  is situated on the line  $a_3b_3$ , and similarly a point  $c_4$ , additive effect of  $b_1$  and  $a_3$  will be situated on the line  $a_4b_4$ . We obtain, therefore, a series of straight and parallel isochrones or lines of equal incubation time.

If there is any antagonism in the action of substances  $a$  and  $b$  the

isochrones will tend to show protrusions in the direction of maximum antagonism (Fig. 2).

The angle  $\alpha$  is a measure of the antagonism  $\text{arc.tg } \frac{b_{\text{ant}}}{a_{\text{ant}}}$  in which  $b_{\text{ant}}$  and  $a_{\text{ant}}$  represent the antagonistic concentrations of the substances  $a$  and  $b$ , if we assume that this antagonism, in the range of concentrations of  $a$  and  $b$  is independent of the concentration. In the present paper we will find that, over small ranges of concentrations

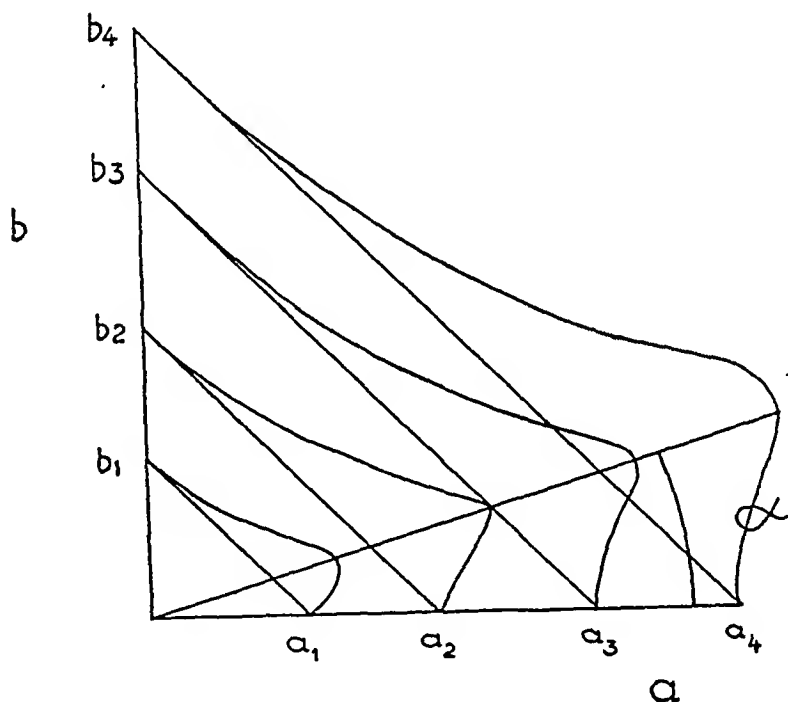


FIG. 2. Influence of salts  $a$  and  $b$  on incubation time (dotted lines). Antagonism, measured by  $\text{tg } \alpha$ .

this happens to be the fact; otherwise the bundle of curves would be like those depicted in Fig. 3.

Fig. 4 shows the application of the principle to cultures from Marina at pH 9 in various molarities of NaCl. When there are very large amounts of Mg required to detoxify the calcium, the determination loses much of its accuracy. As a matter of fact, there is a great deal of arbitrariness in the construction of the "contour" lines. Still, it seemed advisable to mention the matter here as it gives an approxi-

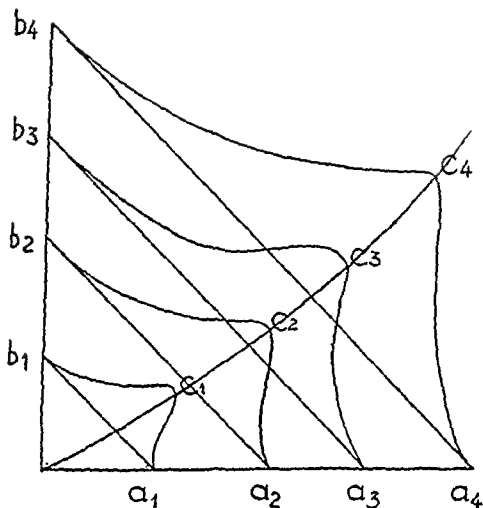
FIG. 3. Antagonism of substances *a* and *b*. Non-linear.

TABLE III

|                   | Mol Na | Number of cultures | Averages |        | Average | Mg/Ca from incubation time |
|-------------------|--------|--------------------|----------|--------|---------|----------------------------|
|                   |        |                    | Mol Mg   | Mol Ca | Mg/Ca   |                            |
| Marina pH 9       | 1      | 13                 | 0.17     | 0.08   | 2.1     | 4.0                        |
|                   | 2      | 12                 | 0.18     | 0.07   | 2.6     | 6.0                        |
|                   | 3      | 8                  | 0.14     | 0.018  | 7.4     | 10.0                       |
|                   | 4      | 7                  | 0.086    | 0.015  | 5.8     | 20.0(?)                    |
| Sand Springs pH 9 | 1      | 9                  | 0.1      | 0.09   | 1.1     | 4.5                        |
|                   | 2      | 6                  | 0.1      | 0.005  | 20.0    | 6.0                        |
|                   | 3      | 4                  | 0.075    | 0.0075 | 10.0    | 9.0                        |
|                   | 4      | 3                  | 0.033    | 0.0067 | 4.9     | 18.0                       |

mate measure of antagonism and also because it might be a useful method for the study of this phenomenon.

As the incubation time at pH 6 is long and rather uncertain and as the number of developing cultures at pH 6 was so small, only the results at pH 9 are mentioned here.

Even if we take the averages of the magnesium-calcium ratio of the cultures that did develop, we see that there seems to be an antagonism. The last column in Table III shows that more magnesium is required

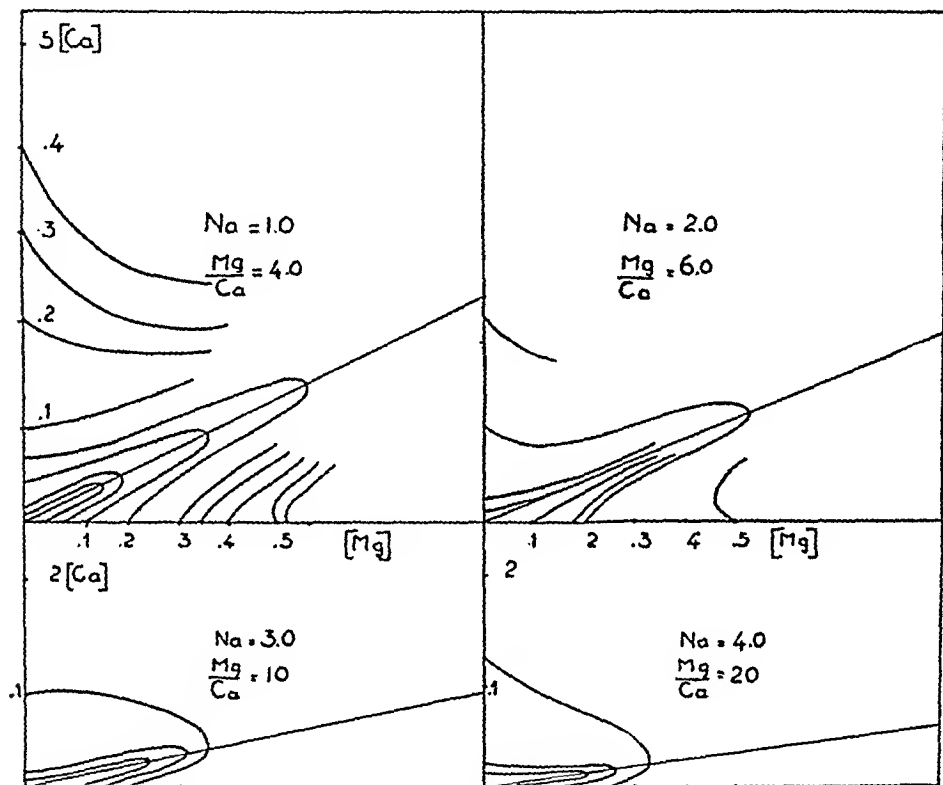


FIG. 4. Antagonistic action of calcium (ordinates) and magnesium (abscissae) of *Dunaliella viridis* in various molalities of sodium (1-4).

to detoxify the calcium at higher concentrations of sodium chloride. Now, when we consider Table I again we see that when sea water evaporates, the magnesium-calcium ratio increases with the increasing NaCl content of the brine. This ratio, plotted as a function of NaCl concentration, shows discontinuities at the points where calcium carbonate and gypsum precipitate. Fig. 5 shows that the antagonisms, as measured in *Dunaliella*, vary with the NaCl concentration in the

same range as the  $Mg/Ca$  ratio in sea water varies with its  $NaCl$  concentration.

Inasmuch as magnesium *per se* seems toxic to *Dunaliella*, the upper concentration limit seems to be determined by the calcium concentrations in its environment.

Now, neither in the natural brines from Sand Springs nor in the natural brine from Marina, could we detect any calcium or magnesium

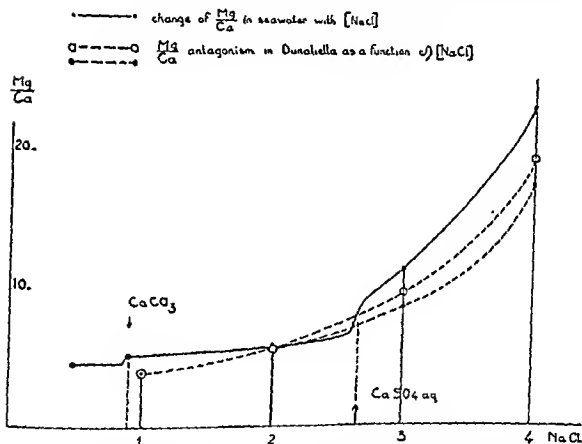


FIG. 5. Ratio  $Mg/Ca$  as a function of  $Na$ -concentration in seawater-evaporates in relation to the behavior of *Dunaliella* toward magnesium and calcium.

with the usual chemical methods. Still, both strains behaved as if they were adapted to the chemical environment created by the evaporation of sea water.

It is, therefore, not strictly permissible to speak about "calciphobic" or "calciphilic"; only the study of the interplay between all the cations concerned (and in other organisms, also the anions) determine the character of an organism.

At the hydrogen ion concentration studied (pH 9), the calcium and

magnesium were still in solution. The margin for precipitation for the calcium was narrow, as certain cultures, despite the heavy phosphate buffering, might well have transgressed pH 9.2 and so caused the Mg/Ca ratio to be upset. In no instance could large increases in pH be demonstrated. (Some cultures showed even a slight decrease.) The fact that calcium and magnesium proved to be so very toxic in acid media cannot be accounted for by precipitation of these metals in the alkaline cultures. From Table I we may learn one other thing. The absolute amount of magnesium increases very rapidly above any concentration in which, at corresponding molarities of NaCl, we observed development within 6 months. This confirms the practical

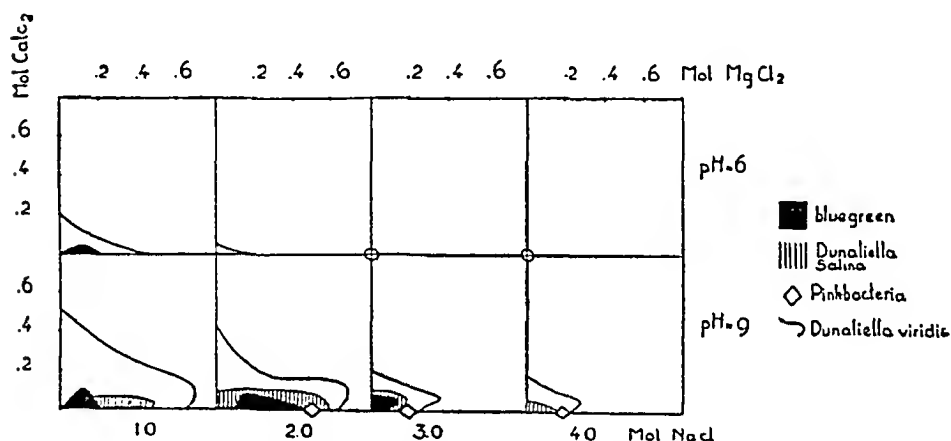


FIG. 6. Occurrence of various organisms as dependent upon saline environment and pH.

experience of the "briner", who sees in the dying of *Dunaliella* and the appearance of red bacteria the point at which most of the calcium is precipitated and the brine is ready to "corn".

The above considerations pertain to *Dunaliella viridis*. Fig. 6 illustrates the limits of its development in the various media. The development of the "red cysts", blue-green algae, and red bacteria is also indicated. As the appearance of red bacteria is bound to organic material, not much importance must be attached to the latter data.

Inasmuch as the light intensity might have been insufficient for the development of the hematochrome in the swarmers of the orange strain of *Dunaliella*, the swarmers probably represented both strains, as the development of orange cysts showed.

The blue-green alga was rather friendly toward calcium in the lower NaCl concentrations; but it became very sensitive to it at higher concentrations, and also to the magnesium, so that, at 4 molar NaCl, it only developed in a culture without bivalent metal.\* The diagram shows that as far as environment is concerned, *Dunaliella* is the most plastic in the green form. The orange form is more sensitive to calcium, not more sensitive to magnesium. The red bacteria, if we may

TABLE IV

|                                       | Marina<br>swarmers after<br>6 months | Pal-<br>mella | Germi-<br>nating<br>zygotes | Orange<br>cysts | Blue-<br>green | Blue-<br>green<br>exclu-<br>sively | Red<br>bacteria |
|---------------------------------------|--------------------------------------|---------------|-----------------------------|-----------------|----------------|------------------------------------|-----------------|
| Number of cultures.....               | 20                                   | 40            | 8                           | 21              | 25             | 6                                  | 3               |
| Days, average incubation<br>time..... | 47                                   | 40            | 78                          | 46              | 43             | 77                                 | 35              |
| Average pH. ....                      | 8.5                                  | 8.3           | 7.8                         | 8.3             | 8.2            | 7.0                                | 9.0             |
| Aver. [Na] .....                      | 1.94                                 | 2.06          | 1.96                        | 2.30            | 1.56           | 1.50                               | 3.0             |
| Aver. [Mg] .....                      | 0.20                                 | 0.14          | 0.28                        | 0.26            | 0.13           | 0.12                               | 0.30            |
| Aver. [Ca] .....                      | 0.045                                | 0.031         | 0.026                       | 0.021           | 0.012          | 0.040                              | 0               |
| Aver. $\frac{[Mg]}{[Ca]}$ .....       | 4.4                                  | 4.5           | 10.8                        | 12.4            | 10.8           | 3.0                                | 0               |
| Aver. size. ....                      | 6 $\mu$ x 8 $\mu$                    |               |                             | 11.7 $\mu$      |                |                                    |                 |
| Aver. total molarity....              | 2.59                                 | 2.51          | 2.50                        | 2.77            | 1.81           | 1.66                               | 3.30            |

conclude anything from the meagre data, are most calciphobic. The *Dunaliella* strain from Sand Springs was definitely more calciphobic especially in the higher NaCl concentration.

As it is impossible to get a clear impression from the perusal of over 200 records, a summary of the results is given in Tables IV and V. The averaging of the data obscures many valuable details; still, several conclusions may be drawn from the figures on the table.

\* It is probable that the *Microcoleus* used as "ground cover" in the Mediterranean and Portuguese salterns is more adaptable than the blue-green studied in these experiments.

(a) The *red bacteria* require high salinity, pH, and magnesium. They do not develop in cultures containing calcium. The short incubation time means a copious supply of disintegrating algae.

TABLE V

|  | Sand Springs<br>swarmers after<br>6 months | Palmella | Germi-<br>nating<br>zygotes | Fungus |
|--|--|----------|-----------------------------|--------|
| Number of cultures. ....                 | 16   | 34       | 3                           | 9      |
| Days, average incubation time. ....      | 22   | 40       | 70                          | 52     |
| Average pH. ....                         | 8.6  | 8.3      | 7.0                         | 8.3    |
| Aver. Na. ....                           | 1.80                                       | 2.03     | 1.00                        | 1.34   |
| Aver. Mg. ....                           | 0.075                                      | 0.094    | 0.10                        | 0.27   |
| Aver. Ca. ....                           | 0.011                                      | 0.004    | 0.040                       | 0.11   |
| Aver. $\frac{\text{Mg}}{\text{Ca}}$ .... | 6.9  | 2.35     | 2.5                         | 2.5    |
| Aver. size. ....                         | 6.9 $\mu$ $\times$ 9.6 $\mu$               |          |                             |        |
| Aver. total molarity. ....               | 1.89                                       | 2.13     | 1.14                        | 1.72   |

TABLE VI

*Marina. 60 Day Culture. No Mg, No Ca, pH 9*

| Length in $\mu$ | 2M NaCl         | 3M NaCl         | 4M NaCl         |
|-----------------|-----------------|-----------------|-----------------|
| 3.7-4.9         | 12              | 0               | 0               |
| 4.9-6.1         | 10              | 2               | 1               |
| 6.1-7.3         | 10              | 6               | 1               |
| 7.3-8.5         | 29              | 27              | 12              |
| 8.5-9.7         | 6               | 8               | 11              |
| 9.7-10.9        | 3               | 11              | 27              |
| 10.9-12.1       | 0               | 3               | 5               |
| 12.1-13.3       | 0               | 1               | 1               |
| 13.3-14.5       | 0               | 0               | 0               |
|                 | Mean 6.89 $\mu$ | Mean 8.67 $\mu$ | Mean 9.32 $\mu$ |

(b) *Zygotes* of *Dunaliella*, which appear in cultures with slow development, tend to show that this formation sometimes occurs after the vegetative phase.



(c) The formation of *palmella* and *swarmers* was most marked in cultures with rapid development about 2 molar in NaCl and with rather large Mg/Ca ratio. The Sand Springs strain is more sensitive to calcium.

(d) The *orange cysts* (*D. salina* ?) develop in higher molarity of NaCl and require a very high Mg/Ca ratio.

(e) The opposite is true for the *blue-green* and the *fungus*, which developed in lower NaCl concentrations and in a low Mg/Ca.

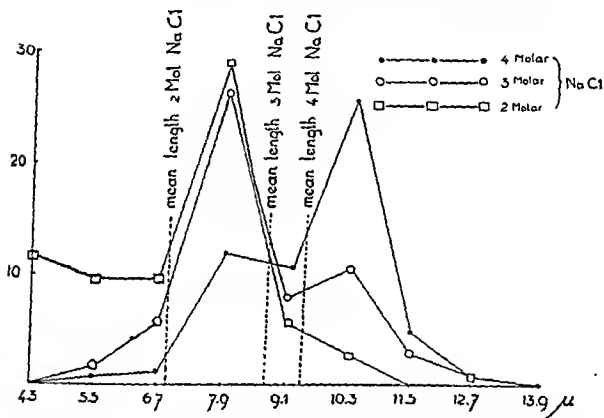


FIG. 7. Frequency-length diagrams of *Dunaliella* in various concentrations of NaCl.

Thus, during the evaporation of sea water, we may predict the environment will become suitable, in succession for: *blue-green algae and fungus* → *Dunaliella viridis*, first *swarmers* and *zygotes*, then *palmellae* → *D. salina* → *red bacteria*.

It has been claimed that *Dunaliella* *swarmers* become smaller as the concentration of the milieu increases.

From the concepts developed in this paper, this would not necessarily follow.

Actual measurements showed that the size of the *swarmers* seems to

vary more or less due to frequency of division, as the recently divided swarmer is, of course, smaller than the mother-cell (see Baas-Becking and Baker (1)). It might be that the bimodal curves in Fig. 7 actually represent two strains of *Dunaliella*; although, in these cultures, no orange cysts appeared. On either of the two assumptions, there seems to be no reason to assume that the size of the flagellate depends upon salinity.

In both Sand Springs and Marina strains, it could be observed that a high calcium content of the medium influenced the size of the swarmers, so that, in cultures containing more than 0.2 mol calcium, the cells were very small ( $\pm 5\mu$  long). Neither the magnesium nor the sodium concentration seemed to have a great effect upon size.

Actual transfer experiments, measuring change in size of definite cells, are difficult. There is no doubt that such experiments are desirable.

#### SUMMARY AND CONCLUSIONS

1. *Dunaliella viridis* Teodoresco thrives equally well in solutions of NaCl 1 to 4 mol and pH 6 to 9.

2. The organism is sensitive to calcium and magnesium, especially in acid medium.

3. Calcium and magnesium are antagonistic. In a molar solution of NaCl the antagonistic relation Mg:Ca is 4 to 5. In a 4 molar solution of NaCl the proportion becomes many times as great (20:1).

4. Although the strains used in this investigation did not occur in sea water concentrates, the increase in the antagonistic ratio Mg:Ca in which they can live closely paralleled the changes in this ratio which take place when sea water evaporates.

5. The other organisms which occurred in the cultures each show a specific relation to Ca and Mg.

6. The size of the cells of *Dunaliella* does not decrease with increasing NaCl content.

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